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Integrated Enzymatic Treatment System

By

Xiaojian Mao

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

In

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Abstract

An integrated enzymatic wastewater treatment system, which includes *coprinus cinereus* peroxidase (CIP) production, purification, and batch and plug flow reactors, was established to investigate the use of *coprinus cinereus* peroxidase to remove phenolic compounds from the aqueous solution.

The production of CIP using a Bioflo 110 fermentor was demonstrated feasible and the maximum production of CIP was almost 60 U mL^{-1}.

The concentration of CIP using Plate-and-Frame module and Spiral Wound module were studied. The Spiral Wound filter module was demonstrated feasible and at least 10 fold concentration can be achieved. Furthermore, COD in the crude enzyme solution was removed 83% after the concentration.

The purity of CIP was demonstrated to have no effects on the removal of phenol from the aqueous solutions. The polyethylene glycol (PEG) additive has no positive effects on the phenol removal from un-buffered solutions. In the plug flow reactors, step addition of CIP and hydrogen peroxide can improve the phenol removal efficiency 15% and 7%, respectively. Step addition of CIP can achieve higher removal rates.

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List of Symbols and Abbreviations

4-AAP		4-aminoantipyrine
A	cm^2	area of the membrane
A'		Arrhenius constant
$[AH_2]_0$	mM	initial phenol concentration
ARP		arthromyces ramosus peroxidase
C	(mmol dm^{-3})	dissolved oxygen concentration in the bulk of the liquid
C_b	U mL^{-1}	concentration of the species of interest in the bulk solution
Ccrit		critical concentration
CFF		Crossflow filtration
CIP		coprinus cinereus peroxidase
CMP		coprinus macrohizus peroxidase
COD		chemical oxygen demand
C_p	U mL^{-1}	concentration of the species of interest in the permeate
C_{ph}	mM	concentration of phenol
C_s	mmol dm^{-3}	saturated dissolve oxygen concentration
CSTR		continuous stirred tank reactor
C_w	U mL^{-}	concentration of the species of interest at the membrane wall
dC /dt	mmol dm^{-3} h^{-1}	change in oxygen concentration with time
DCP		2,4-dichlorophenol
DO		dissolved oxygen
D_z		diffusion coefficient

E	$U\ mL^{-1}$	enzyme dose required to achieve a specific removal with or without additives
E_a		energy of activation
Ei		compound I
Eii		compound II
Eiii		compound III
HRP		horseradish peroxidase
$(HRP)_{min}$	$U\ mL^{-1}$	minimum HRP dose required to achieve at least 95% removal
HRT		hydraulic retention time
J	$mL\ min^{-1}\ cm^{-2}$	filtrate flux
k		reaction rate constant
K_{La}	h^{-1}	the overall mass transfer coefficient
MWCO		molecular weight cut off
NaPP		monobasic-dibasic sodium phosphate
OTR		oxygen –transfer rate
PEG		polyethylene glycol
[PEG]	$mg\ mL^{-1}$	minimum amount of PEG for maximum protection of HRP
$(PEG)_{min}$	$g\ L^{-1}$	minimum PEG dose under the minimum dose of HRP($U\ mL^{-1}$) for at least 95% phenol removal
PFR		plug flow reactor
$(\Delta P)_{TM}$		Transmembrane pressure differential
PTFE		Polytetrafluoroethylene
PVDF		Polyvinylidine-difluoride

Q_{O2}		oxygen on specific oxygen uptake rate
Q_p	mL min^{-1}	permeate flowrate
R		solute rejection coefficient
R'		gas law constant
R_{cp}	m^{-1}	resistance of the concentration polarization layer
R_f	m^{-1}	resistance of the fouling layer
r_M	mmol s^{-1}	rate of oxygen used by the microorganisms
R_m	m^{-1}	intrinsic resistance of the membrane
R_{tot}	m^{-1}	resistance in total
SBP		soybean peroxidase
SBP_{min}	U mL^{-1}	minimum SBP required
t	h	Time
T		absolute temperature
TFF		tangential flow filtration
TMP		Transmembrane pressure differential
X		desired conversion of phenol
z	cm	perpendicular distance above membrane surface
δ	cm	thickness of the boundary layer
λ	U mL^{-1} mM^{-1}	enzyme dose coefficient with or without additives
μ_p	N s m^{-2}	viscosity of the permeate

1. Introduction

Aromatic compounds (phenol, anilines and their derivatives), are present in the wastewaters of numerous industries including petroleum refining, coal conversion, plastics, resins, textiles, wood preservation, iron and steel manufacturing, dyes and organic chemical manufacturing, timber, soaps and detergents, paving and roofing, ore mining and dressing. Many of these are toxic and some are known or suspected carcinogens (Klibanov 1982). The typical concentrations of phenol found in industrial waste streams range from 1 to 10 mM (100 to 1000 mg L^{-1}) (Wu et al. 1993). The maximum acceptable concentration (MAC) of 2,4,6-trichlorophenol in drinking water is 5 μg L^{-1} (Community water supplies, CCME, 1999). The concentration in the freshwater to protect the aquatic life is 4.0 μg L^{-1} (Canadian Water Quality Guidelines for the Protection of Aquatic Life, CCME, 1999). Therefore it is very important to remove the aromatic compounds from the contaminated industrial aqueous streams before discharge into any water body.

Existing treatment methods for removing these kinds of chemicals from the industrial wastewaters include microbial and chemical oxidation, adsorption on activated carbon, extraction, incineration, electrochemical techniques and irradiation (Klibanov 1982). These methods are feasible and useful, however, they entail some difficulties such as high sludge production, incomplete removal, relatively low efficiency.

At the beginning of the 1980s, A. M. Klibanov and his coworkers (1980) proposed an enzymatic method to remove the phenolic matter from wasterwaters using horseradish peroxidase (HRP). Since then, a number of researchers have studied this method and

determined its ability to successfully remove the aromatics from different industry wastewaters (Aitken et al. 1994; Buchanan and Han 2000; Buchanan and Nicell 1997; Buchanan and Nicell 1998; Heck et al. 1992; Ikehata and Buchanan 2002a; Ikehata and Buchanan 2002c; Klibanov 1982; Klibanov et al. 1980; Klibanov et al. 1983; Nicell et al. 1993a; Nicell et al. 1995).

Peroxidase, one class of enzyme, has oxidative properties and can catalyze effectively the oxidation of reducing substrate, such as phenol and other aromatics (Dunford 1999). Thus, peroxidases have many potential applications in the water and wastewater treatment field, especially in industrial wastewater treatment (Aitken 1993; Maloney et al. 1984; Nicell et al. 1993a). Horseradish peroxidase (HRP) and peroxidases from Coprinus cinereus (CIP), Coprinus macrorhizus (CMP), Arthromyces ramosus (ARP) and soybean (SBP) have been studied and determined to be effective in removing aromatic compounds from wastewater (Al-Kassim et al. 1994a; Buchanan and Han 2000; Buchanan and Nicell 1998; Flock et al. 1999; Ikehata and Buchanan 2002b).

The biggest obstacle to applying the enzymatic method to practical situations is the high enzyme cost. Some researchers have demonstrated that chemical additives can inhibit the peroxidase inactivation and improve the enzyme lifetime, thus decreasing the operation cost. The peroxidase inactivation is mostly as a result of interactions between the enzyme and free radicals (Klibanov et al. 1983), and the enzyme and the end-product polymers (Nakamoto and Machida 1992). Inhibition of the enzyme by hydrogen peroxide (or reversible inactivation) results from the formation of compound III, which spontaneously reverts to the native enzyme (Buchanan and Nicell 1997). In the case of

HRP, formation of a permanently inactive enzyme form designated P-670 has been observed in the presence of excess hydrogen peroxide.

In order to overcome this kind of shortcoming and prolong the lifetime of the enzyme, chemical additives such as high molecular weight polyethylene glycol (PEG), protein, gelatin, bovine serum albumin and polyvinyl alcohol has been studied. PEG was found to be a practical effective chemical additive because it is not toxic to humans. Using the chemical additives can not avoid the inactivation completely. Also the addition of PEG will increase the organic load.

Some researchers proposed using crude enzyme to treat the aromatic compounds from wastewater and demonstrated that is feasible and cost-competitive. A crude enzyme extracted from the minced horseradish roots was demonstrated to have the same removal efficiency as the commercial purified enzyme, so the efficiency of phenol removal by the enzymatic method does not depend on the purity of the enzyme (Alberti and Klibanov 1982). Dec et al. (1994) have also demonstrated that the crude enzyme from minced horseradish, potato, and white radish are effective to remove the phenols. The crude soybean enzyme and Coprinus *cinereus* peroxidase (CIP) have been proved to be more effective in removing aqueous phenols than purified enzyme (Flock et al. 1999; Masuda et al. 2001a).

Therefore, using crude enzyme to treat wastewater containing aromatic compounds seems attractive and cost effective. However, very few studies have been carried out to determine a cost effective production method. Ikehata and Buchanan (2002c) speculated that extracellular fungal peroxidases are likely to have a great potential for application to wastewater treatment because of their relatively simple production and separation

procedures compared with plant enzymes that are secreted within the tissue of the organisms.

1.1 Objective

The objective of this project is to integrate low cost enzyme production into the treatment of aromatics from industrial wastewaters and to demonstrate the feasibility and effectiveness of the integrated enzymatic system in removing aqueous phenols from the wastewater.

1.2 Scope

The scope of this research comprises:

- Testing the feasibility of the production of CIP by UAMH 4103 *Coprinus cinereus* strain grown in a Bioflo 110 fermentor;
- Using a membrane process to separate the CIP from the fermentor broth and testing membrane separation for
    - Fermentor organics separation, and
    - Enzyme solution purification; and
- Operating and optimizing a tubular plug-flow reactor for phenol removal.

2. Literature Review

2.1 Enzymatic Wastewater Treatment

Conventional biological processes may not be reliable as a final treatment process to meet discharge standards for specific compounds because they often have difficulty in removing toxic pollutants consistently to low levels and they also may be susceptible to inhibition by one or more compounds in the waste such as phenols (Aitken 1993). In addition, industry effluents can vary greatly in flowrate, concentration of the pollutants, temperature, and pH. Biological processes need large volume ponds for equalization and long retention time for phenol removal (Nakamoto and Machida 1992). Most physicochemical treatment processes are more economical for the treatment of dilute wastewaters and are often used as polishing steps. Chemical oxidation processes can become prohibitively expensive for highly strong wastes even though the target pollutants might only be present in low concentrations (Aitken 1993).

These disadvantages plus more and more restricted effluent standards and increasing number of industries, spurred interest in enzymatic treatment methods for aromatic removal.

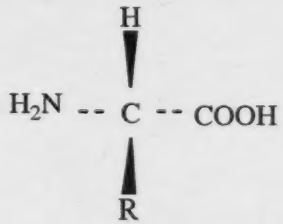
Compared with the conventional biological treatment, the enzymatic method can apply to a broad range of aromatic compounds, including those which are biorefractory or toxic to microbes (Kauffmann et al. 1999; Klibanov 1982), moreover, co-precipitation with the easily removed phenols and aromatic amines can greatly enhance the enzymatic removal of those that alone have low removal efficiency (Kauffmann et al. 1999; Klibanov 1982). The enzymatic method can operate at high and low concentrations of contaminants and

cope with shock loading effects; it can also operate over wide ranges of temperature, pH and salinity. Another advantage is much lower production of sludge. The potential advantages of the enzymatic method over chemical/physical processes are operation under milder, less corrosive conditions in a catalytic manner, reduced consumption of oxidants or adsorbent materials such as charcoal, removal of the trace level organic compounds and organics not removed by existing chemical/physical processes (Nicell et al. 1993a). Kauffmann et al. (1999) believed that enzymatic wastewater treatment opens a new avenue for the treatment of effluent streams that contain hazardous or xenobiotic organic pollutants.

2.2 Enzymes

2.2.1 Enzyme structure

Enzymes are globular proteins. The monomeric building blocks of proteins are the α-amino acids, whose general structure is:



The acid (- COOH) and base (- NH_2) groups of amino acids can ionize in aqueous solution. The amino acid is positively charged at low pH and negatively charged at high pH. At an intermediate pH, which is called isoelectric point, the amino acid has no net charge. At its isoelectric point, an amino acid exhibits minimum solubility (Bailey and Ollis 1986).

There are four structural levels of proteins if the proteins have more than a single chain: primary structure, secondary structure, tertiary structure, and quaternary structure. The primary structure is the sequence of amino acid residues. Every protein has not only a definite amino acid content but also a unique sequence (Bailey and Ollis 1986).

Secondary structure is the structural configuration of the amino acid residues, which are close neighbors in the linear acid sequence. Helices and sheets are the two general forms of secondary structure. The α-helical structure is easily disturbed due to the relative weakness of individual hydrogen bonds. Thus, protein can lose this structure in aqueous solution (Bailey and Ollis 1986).

Tertiary structure is the complete three-dimensional configuration of a protein. Several weak interactions, such as ionic effects, hydrogen bonds, and hydrophobic interactions between nonpolar R groups, primarily determine how the chain is folded or bent to form the tertiary structure of the protein. These weak interactions are easily disrupted by changing the environmental conditions of the protein, like the temperature, pH, ionic strength, physical forces, and addition of organics. Denaturation will happen when the environmental conditions of a protein change sufficiently from its normal biological environment. Relatively small changes such as solution temperature or pH may denature the protein (Bailey and Ollis 1986). Therefore, an enzymatic treatment system should operate at certain range of pH, temperature and ionic strength to optimize the removal.

Quaternary structure is how the chains fit together in the molecule when the protein consists of more than one polypeptide chain. Enzymes (usually have the –ase suffix) are oligomeric and possess quaternary structure. The weak interactions, which produce the

tertiary structure, are believed to stabilize the quaternary structure (Bailey and Ollis 1986).

2.2.2 Enzyme characteristics

Enzymes are bio-catalysts. They can increase the rate of a chemical reaction. However, they do not undergo a permanent chemical change themselves, and they do not affect the reaction equilibrium. In the catalyzed reaction, the catalyst actually takes part in and is altered during the reaction, but is returned its native form in the final step of the reaction. During the catalyzed reaction, the catalyst will change the path of the reaction, decrease the reaction's activated energy, and thereby increase the reaction rate.

Enzymes are very powerful catalysts. Firstly, they are very efficient, catalyzing reactions often 10^8 – 10^{11} times more rapidly than the corresponding nonenzymatic reactions. Compared with the uncatalyzed reactions, these rates can be achieved even though the enzyme catalyzed reactions take place under much less extreme conditions, such as temperature, pH, and pressure (Wiseman 1985). Secondly, enzymes are usually very specific in terms of the type of reaction catalyzed compared with chemical oxidants. Thirdly, enzymes are naturally subject to a number of controls both in terms of the gross control of how fast they are synthesized and degraded, and in the fine control of their activity by the binding of small molecular modifiers which can increase or decrease the activity of the enzymes (Wiseman 1985). With respect to some other enzyme classes, peroxidases enzyme can catalyze a much greater variety of reactions.

The widely accepted mechanism of enzyme catalyzed reaction is enzyme-substrate complex and active sites. The substrate binds to the enzyme active site to form an

enzyme-substrate complex. At the active site, the reaction is carried out and the products are formed (Bailey and Ollis 1986).

Enzyme activity depends significantly on the pH value of the medium. Activity increases to a maximum with increasing pH value and then drops to zero in the alkaline region (Bisswanger 2002).

The rate of the enzyme-catalyzed reaction increases with temperature by a factor of 2 to 3 per 10 °C. After the optimal temperature, the reaction rate will decrease rapidly (Bisswanger 2002).

Enzyme inhibition is an important topic in the study of enzyme kinetics, mechanisms and application. Enzyme inhibition can be classified into two categories: reversible and irreversible inhibition (Bisswanger 2002). Reversible inhibition also can be divided into: competitive, non-competitive and excess substrate inhibition (Wiseman 1985). Taylor (2002) classified the enzyme inhibitor as: 1) rapid reversible inhibitors; 2) slow-binding, reversible inhibitors; 3) rapid, tight-binding inhibitors; 4) slow, tight-binding inhibitors; and 5) irreversible inhibitors.

2.2.3 Peroxidase Enzyme

Peroxidases (donor: H_2O_2 oxidoreductase; EC. 1.11.1.7) are enzymes. The peroxidase superfamilies include plant peroxidase superfamily, animal peroxidase superfamily and catalase superfamily (Dunford 1999). On the basis of sequence homologies, Welinder (1992) proposed three families of peroxidases which belong to the plant peroxidase superfamily: peroxidases of prokaryotic origin, secreted fungal peroxidases, and classical secretory plant peroxidases. HRP and SBP belong to classical secretory peroxidases. *C.*

cinereus peroxidase (CIP), *C. macrorhizus* peroxidase (CMP) and *A. ramosus* (ARP) belong to secreted fungal peroxidases. The peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus* were found to be similar in their primary structure (Kjalke et al. 1992).

2.2.4 *Coprinus cinereus* Peroxidase

It has been known since 1900 that phenols can be oxidized by the peroxidase system and peroxidases can be produced by plants, animals, fungi, and bacteria (Saunders et al. 1964). The first reports on *Coprinus cinereus* peroxidase appeared in the patent literature in 1986. The common name for the source is the black inkcap mushroom, from the Basidiomycete family of fungi. *Coprinus cinereus* peroxidase was proposed as an alternative for horseradish peroxidase for various commercial assays because of its stability and accessible active site. Another name for *Coprinus cinereus* is *Coprinus macrorhizus* (Dunford 1999).

Coprinus cinereus peroxidase (CIP) was found to be similar to horseradish peroxidase in many of its properties. CIP contains one ferriprotoporphyrin IX as its prosthetic group, it is a glycoprotein, and it forms conventional compounds I and II (Dunford 1999). Typically, there are at least five different oxidation states of the prosthetic group of a peroxidase (Dunford 1999). CIP is an acid protein, with a pI value of 3.5. However, the pKa value of the active site group in the *Coprinus cinereus* enzyme is 5.1±0.2, much higher than that in horseradish peroxidase (Dunford 1999).

The molecular weight of *Coprinus cinereus* peroxidase is 42,000 Daltons (Morita et al. 1988). This enzyme's high specific activity and substrate profile are very similar to those

of classical plant peroxidases with which CIP shares less than 10 to 16% sequence identity.

2.3 Production of Fungal Peroxidase

Peroxidases are mainly produced from plants such as horseradish roots and soybeans, the production is affected by the culturing conditions such as weather, therefore it is very difficult to produce in large amount regarding the industrial utilization (Sakurai et al. 2002). The production of peroxidase from microorganisms has attracted research interest. Extracellular fungal peroxidases' production has some advantages such as relatively simple production and separation compared with other plant and intracellular fungi (Ikehata and Buchanan 2002c). Peroxidase production by *Coprinus cinerus* seems to get some attention regarding phenol removal from industrial wastewater streams.

Culturing media were used to inoculate the fungus in a shaking flask and high peroxidase production was achieved (Morita et al. 1988; Shinmen et al. 1986). This indicates that it is possible to use a fermentor to culture *Coprinus cinereus* species to produce CIP.

The advantage of using microorganisms as potential enzyme sources is that it is easy to get high enzyme production by environmental and genetic manipulation compared with plant and animal sources. The other advantages are: 1) economical enzyme fermentation on a large scale; 2) simple screening procedure; and 3) flexibility with respect to desired operation conditions in the reactors (Stanbury et al. 1995; Wang et al. 1979). Furthermore, the recombinant DNA technology can enable enzymes of animal origin to

be synthesized by microorganisms (Stanbury et al. 1995). *Coprinus cinereus* peroxidase is an extracellular peroxidase from fungi. It is easy to separate from the biomass.

2.3.1 Factors important to enzyme production

2.3.1.1. Strain selection

It is very important to select the most active strain available. The major requirement in screening is the efficiency so that rapid examination of a larger number of strains is feasible (Wang et al. 1979). Ikehata and Buchanan (2002c) have investigated the production of extracellular peroxidase by 25 strains of *Coprinus* species for the purpose of its application to the removal of phenolic and other aromatic compounds from industrial waste streams. *C. cinereus* UAMH 4103 was determined to yield the highest production of peroxidase of the sources tested. Korus et al. (1991) attempted to improve peroxidase production by selecting better strains, using genetic manipulation and optimization of bioreactor design and operation. They found recombinant *S. lividans* TK64.1 can increase 72% in maximum enzyme activity compared with *S. viridosporus* T7A.

2.3.1.2. Fermentation Optimization

Once the microorganism strain has been chosen, the operation of the fermentor must be optimized to maximize the cell growth and the enzyme production. The important optimization factors include the medium composition, temperature, pH and oxygen transfer. Faith et al. (1971) pointed out that the addition of surfactants is important, especially with extracellular enzyme because it increased the yields of many enzymes.

The mechanism by which surfactants increased enzyme secretion was not known, but there were some possible explanations: 1) Surfactants may be expected to accumulate at the cell membrane and thereby increase the "leakiness" of the cell membrane; 2) Surfactants may protect the enzyme from inactivation. Nonionic surfactants are preferred over anionic and cationic surfactants because the latter are more toxic to the fungi.

Growth inhibition might result from combined effects of temperature exposure, shearing, pH, aeration, proteolytic attack, ultrasound, and substrate and product limiting concentrations (Korus et al. 1991).

a. Medium composition

Microorganisms require carbon, hydrogen, oxygen, nitrogen, phosphate, sulfur and trace minerals as elements for cell biomass and as energy for biosynthesis and cell maintenance. Typical value for C, H, O, N and S as a percentage of dry cell weight are 45, 7, 33, 10 and 2.5%, respectively. Trace elements such as Cu, Mn, Co, Mo, B and possibly other metals may also be required, depending on the water source. Some microorganisms may need amino acids, vitamins or nucleotides (Ward 1989). Stanbury et al. (1995) showed that the value for C, N, P, S, as a percentage of dry cell weight of fungi are 40-63, 7-10, 0.4-4.5 and 0.1-0.5 respectively. Korus et al. (1991) has determined that it is not necessary to add additional carbon source such as sucrose, cellobiose, lactose for the highest enzyme activity and good cell growth when the medium is composed of 0.25% (w/v) yeast extract and 0.1% (w/v) each of asparagine, glutamic acid, and proline in the 100 mL basal medium which contains 5.3 g of Na_2HPO_4, 1.98 g of KH_2PO_4, 0.2 g

of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.05 g of $CaCl_2 \cdot 2H_2O$, and 1 ml of a trace element solution. Higher concentrations of amino acids reduced enzyme production.

In the laboratory scale, Morita et al. (1988) used 3% glucose, 2% yeast extract, 0.2% KH_2PO_4, 0.005% $MgSO_4 \cdot 7HO$, and 0.05% $CaCl_2 \cdot 2H_2O$ as the culturing medium produce CIP. Ikehata and Buchanan (2002c) and Shinmen et al. (1986) used 1% glucose, 0.5% peptone, 0.3% malt extract and 0.3% yeast extract as the culturing medium to produce CIP.

Usually, the medium in the industrial fermentation should: 1) produce maximum desired production; 2) produce least by-products; 3) permit maximum production rate of the desired product; 4) keep consistent quality and be readily available; and 5) minimize the problems during medium making, sterilization and production process (Stanbury et al. 1995).

b. Temperature

Like all chemical reactions, microbial growth and product formation are influenced by temperature. However, the optimum temperature for growth and product formation is not necessarily the same and must be examined separately. The microorganisms can be divided into psychrophiles (<20 °C), mesophiles (30-35 °C) and thermophiles (>50 °C). Most microorganisms will only grow over a temperature range of 20-30 °C. As temperature is increased towards the optimal growth temperature, the growth rate approximately doubles over a 10 °C range. Above the optimum growth temperature, growth rate declines rapidly with increasing temperature (Wang et al. 1979). Sakurai et al. (2002) have grown shake flask cultures in the temperature range 25-40 °C, and found

that 35 °C is the optimum temperature for the *Coprinus cinureus* cell production and *Coprinus cinureus* peroxidase production.

c. pH

Medium pH is an important parameter for cell growth and enzyme formation. Most microorganisms will function over a pH range of 3-4 pH units. Usually, bacteria grow at pH 4-8, yeast at pH 3-6, molds at 3-7, and higher eucaryotic cells at 6.5 –7.5.

The pH in the fermentor tends to change during the fermentation. When ammonia is the nitrogen source, the pH tends to fall. If nitrate is the nitrogen source, the pH tends to rise. If organic amino compounds are used for growth, pH tends to rise as the compounds are deaminated. When organic acids such as lactic acid, acetic acid, or pyruvic acid are produced, the pH will change, too. If the predominant reason for pH changes is known, it is possible to get some information on growth and product formation by measuring the addition of acid or alkali to neutralize the change (Wang et al. 1979).

Sakurai et al. (2002) have investigated using rotating disk contactor to produce *Coprinus cinereus* peroxidase, at the initial pH of 7, both the cell production and peroxidase activity can achieve a maximum.

d. High nutrient concentration

In many cases, when the nutrient concentration is increased too much, a region of substrate inhibition occurs. The most likely reason is a dehydration of the cells in such highly concentrated solution. The other reasons may include toxic or inhibitory effects of specific compounds on key enzymes or structural cell compounds (Wang et al. 1979).

Inorganic phosphate concentrations greater than 3-5 mM frequently inhibit the production of plant, fungal and bacterial secondary metabolites (Liras et al. 1990).

e. Oxygen

Most fermentation processes are aerobic requiring provision of oxygen as a raw material. The oxidation of glucose is given in the following reaction:

$$C_6H_{12}O_6 + 6\,O_2 \longrightarrow 6\,H_2O + 6\,CO_2 \qquad (1)$$

When 180 g glucose is oxidized completely, 192 g oxygen is required.

Furthermore metabolic processes are affected by medium-dissolved oxygen and specific oxygen uptake. The relationship between dissolved oxygen and specific oxygen uptake rate follows the typical Michaelis-Menten pattern (Figure 2-1). In general, dissolved oxygen concentrations in excess of a critical concentration (Ccrit) appear to be required to support metabolite production arising from tricarboxylic acid intermediates (Ward 1989).

Oxygen levels significantly affecte the production of lignin peroxidase and manganese peroxidase by *phanerochaete chryosporium*. A high oxygen level is required to produce these enzymes, however, dissolved oxygen (DO) above a critical DO concentration (1.5 fold increase over the saturated concentration with air) inhibited the enzyme production when oxygen was continuously supplied (Choi et al. 2002). The control of oxygen supply is a key factor for the production of CIP because coprinus cineneus strains are also aerobic.

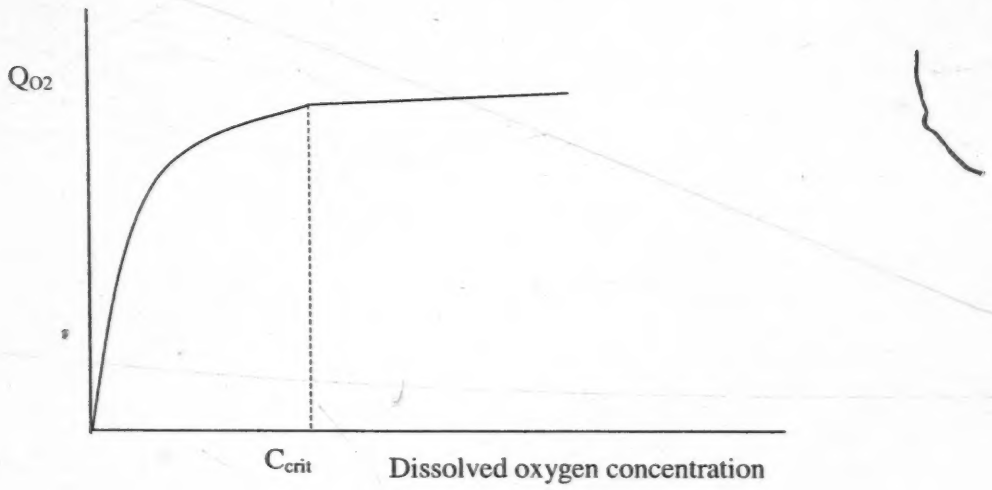


Figure 2-1: Effect of oxygen on specific oxygen uptake rate (Q_{O2}) of microorganism

2.3.1.3. Recognizing the growth cycle peak

If a batch fermentation is used, the enzyme concentration peak must be ascertained, because usually the enzyme will rapidly disappear after the peak (Wang et al. 1979). Enzyme degradation under some sort of metabolic control and enzyme inactivation may be the main reasons for enzyme rapidly disappearing after the peak (Thurston 1972). In continuous culture, a chemostat is quite useful for enzyme production since the formation of many enzymes is controlled by growth rate. Lignin peroxidases producing by *S. viridosporus* or *S. badius* in an agitated slurry cultures with mineral salts solution containing 0.6% yeast extract and 1% (wt/vol) 100/200 mesh ground or 2% (wt/vol) extracted corn lignocellulose for 15 days incubation reached its peak activity in the stationary phase, then the activity dropped and approached a constant level (Adhi et al. 1989).

2.3.2. Fermentor

2.3.2.1 Fermentor components

The main components of a fermentor usually include (Soderberg 1983; Stanbury et al. 1995; Ward 1989): 1) Vessel; 2) Agitator; 3) Inlet and outlet air; 4) Thermometer; 5) Temperature sensor; 6) pH electrode; 7) Oxygen electrode; 8) Other electrode; 9) Addition ports; 10) Inoculum ports; 11) Sampling ports; 12) Oxygen sources; 13) Valves; and 14) Pumps.

The most important part in the agitated fermentor is the drive shaft, its bearings and seals because the liquid in the fermentor is not always evenly mixed due to asymmetric baffling caused by different probes in the fermentor and the off-center addition of medium and other fluids and antifoam or sampling (Nolan and Davies 1990).

2.3.2.2 Sterilization

Fermentor systems have to operate under aseptic conditions to exclude unwanted contaminating organisms. There are many sterilization techniques available, but the only one applied to sterilize the fermentor and its medium is steam. The thermal death of vegetative bacteria follows logarithmic kinetics (Chick 1908):

$$-\frac{dN}{dt} = kN \qquad (2)$$

Since this is logarithmic, sterilization can never be absolute. Sensitive spores take many hours at 100 °C, 15 minutes at 105 °C and approximately 1 min at 120 °C to die. The thermal death of the resistant spores is relatively slow at 105 °C and very rapid at 120 °C (Nolan and Davies 1990).

Sterilization is also an important factor during the fermentation. It not only affects the fermentation environment but also affects the medium quality. Consequently, it will affect the production of the biomass and enzyme.

There are two types of reaction that contribute to the loss of nutrient quality during sterilization (Stanbury et al. 1995):

1) Interactions between nutrient components of the medium. A common occurrence is the Maillard type browning reaction, which results in discoloration of the medium as well as loss of nutrient quality.

2) Degradation of heat labile components. Certain vitamins, amino acids and proteins may be degraded during steam sterilization.

2.3.2.3 Aeration and agitation

The objectives of aeration and agitation are: 1) supply the necessary oxygen to the microorganisms in order to achieve the proper metabolic activities, 2) keep the microorganisms in suspension (Wang et al. 1979).

The oxygen transfer is controlled by the liquid phase mass transfer resistance. It can be described by the following equation (Tchobanoglous et al. 2003):

$$\frac{dC}{dt} = K_{La}(C_S - C) - r_M \qquad (3)$$

Where C = the dissolved oxygen concentration in the bulk of the liquid (mmol dm^{-3}),

t = time (h),

dC /dt =the change in oxygen concentration with time (mmol dm^{-3} h^{-1}) or the oxygen –transfer rate (OTR),

K_{La} = the overall mass transfer coefficient (h^{-1}),

C_s = the saturated dissolve oxygen concentration (mmol dm^{-3}), and

r_M = rate of oxygen used by the microorganisms (mmol s^{-1}).

In conventional fermentation processes, the substrate transfer from the solution to the microorganisms is usually met without difficulty (Ward 1989).

2.3.2.4 Energy transfer

During the growth, the metabolism and mass transfer need some energy to be supplied or removed from the fermentor system. Metabolic energy produced in the metabolism tends to increase the temperature of the fermentation broth. Mechanical agitation and aeration also produce some heat. The balance of heat generation and heat loss will decide if heating or cooling is needed or not. Usually, a heat-exchange system is needed for a fermentation system(Ward 1989).

2.4 Purification of enzyme

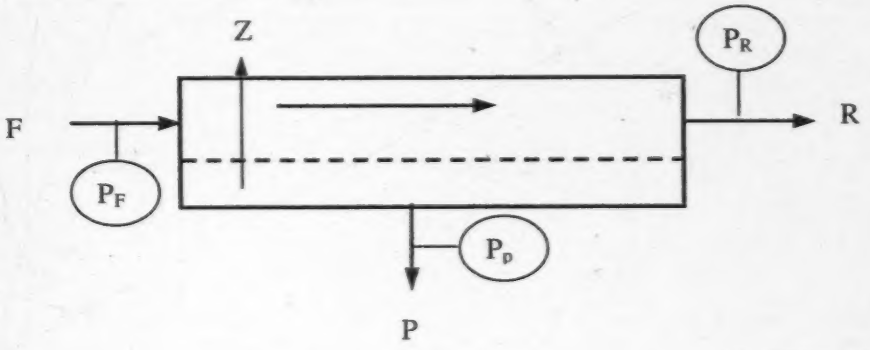
The purification steps for the enzyme usually include biomass/liquid separation by sedimentation, centrifugation, or filtration, followed by the molecular separation by membrane separation, finally crystallization, adsorption, and chromatography may be used (Ladisch 2001).

Ladisch (2001) states that membranes are able to separate colloids, cells, and molecules by microfiltration, ultrafiltration, or reverse osmosis. Microfiltration membranes have nominal pore diameters between about 0.1 and 10 μm and often are used to remove particles such as cells or cell debris from culture suspensions. Ultrafiltration membranes, with nominal pore diameter between about 0.001 and 0.1 μm,

are used to concentrate macromolecules such as proteins, whose molecular weights range from 1 to 500 kDa. Nanofiltration uses charged membranes with pores from 0.0001 to 0.001 μm to remove small molecules, viruses and some harness. Reverse osmosis is used to separate microsolutes (nominal pore diameter< 0.001 μm), such as inorganic salts (Cheryan 1998; Su and Colton 1994; Tchobanoglous et al. 2003)).

Coprinus cinerus peroxidase molecular weight is about 42 k Dalton (Morita et al. 1988). Therefore, ultrafiltration is appropriate to concentrate the peroxidase solution.

Crossflow filtration (CFF), also known as tangential flow filtration (TFF), can be used for many purposes by varying the type of membrane employed. As its parent technology, dead-end filtration, crossflow filtration is a pressure-driven process. The transmembrane pressure differential (ΔP_{TM} or TMP) drives flows of water and some permeable materials through the pores. The general scheme of crossflow filtration is shown in Figure 2-2 (Russotti and Goklen 2001):



Retentate Pressure Drop: $\Delta P_L = P_F - P_R$

Transmembrane Pressure Drop = TMP = $\Delta P_{TM} = (P_F + P_R)/2 - P_P$

Figure 2-2: The general crossflow filtration (CFF) scheme.

2.4.1 Performance Characteristics

Two parameters, filtrate flux (J) and solute rejection coefficient (R), are usually used to characterize the performance of crossflow filtration. They are defined as follows (Su and Colton 1994):

$$J = \frac{Q_p}{A} \qquad (4)$$

where J = Filtrate flux (mL min^{-1} cm^{-2});

Q_p = permeate flowrate (mL min^{-1}); and

A = the area of the membrane (cm^2)

$$R = 1 - \frac{C_p}{C_b} \qquad (5)$$

Where C_p = the concentration of the species of interest in the permeate (U mL^{-1});

C_b = the concentration of the species of interest in the bulk solution (U mL^{-1}); and

R = Solute rejection coefficient

2.4.2 Mechanisms of crossflow filtration

There are a number of models of CFF processes available, but only a few of them can predict the filtration performance of a particular fermentation broth and membrane product. Most of them provide a kind of basis for understanding the underlying processes, designing experiments and interpreting data, and deciding the future researching direction and developments. Modeling CFF is substantially more complex than dead-end filtration and stirred-cell filtration (Russotti and Goklen 2001).

Concentration polarization theory emerged when hydrocolloids, macromolecules (such as proteins) and other relatively large solutes or particles are filtered (Cheryan 1998). The retained species are likely to accumulate on the membrane surface and then form an additional layer, called "gel layer", "cake", or "polarization layer", therefore increase the resistance to the filtration flow. Under those conditions the system operate in the pressure-independent region of operation, where mass transport dominates (Figure 2-3) (Russotti and Goklen 2001).

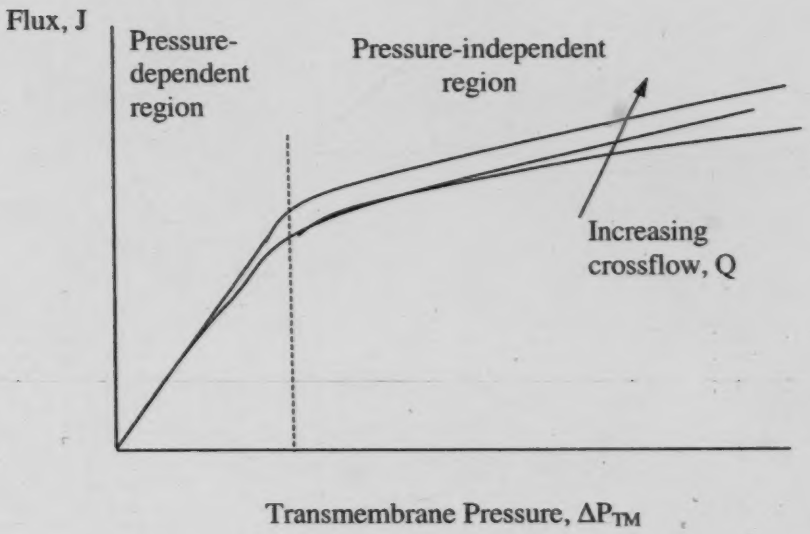


Figure 2-3: Flux dependence on transmembrane pressure and crossflow rate

Mass transfer (Film theory) is one of the simplest and most widely used models for modeling the flux in the pressure-independent region (Cheryan 1998). For concentration polarization model, At steady state, for a specific species that is retained on the membrane surface, convection to the membrane is balanced by diffusion away from the membrane, so (Russotti and Goklen 2001):

$C_p << C_b$

Solving the equation for flux:

$$J C_b = D_z \frac{dc}{dz} \quad (6)$$

then obtain the equation as the following:

$$J = \frac{D_z}{\delta} \ln \frac{C_w}{C_b} = k \ln \frac{C_w}{C_b} \quad (7)$$

Where J = the flux (mL min^{-1} cm^{-2});

C_p = the concentration of the species of interest in the permeate (U mL^{-1});

C_b = the concentration of the species of interest in the bulk flow (U mL^{-1});

C_w = the concentration of the species of interest at the membrane wall (U mL^{-1});

D_z = the diffusion coefficient; and

δ = the thickness of the boundary layer (cm)

z = perpendicular distance above membrane surface (cm)

$$k = \frac{D_z}{\delta} \quad (8)$$

If the entire pressure-flux behavior description is desired, the resistance model is better. This model uses the "resistance-in-series" concept that is common in heat transfer (Cheryan 1998). It correlates flux to ΔP_{tm}, accounting for the effects of the membrane substrate, concentration polarization, and fouling (Russotti and Goklen 2001):

$$J = \frac{\Delta P_{tm}}{\mu_p R_{tot}} \quad (9)$$

$$R_{tot} = R_m + R_f + R_{cp} \quad (10)$$

Where J = the flux (mL min^{-1} cm^{-2});

ΔP_{tm} = Transmembrane pressure drop (kPa);

μ_p = the viscosity of the permeate (N s m^{-2});

R_m = the intrinsic resistance of the membrane (m^{-1});

R_f = the resistance of the fouling layer (m^{-1});

R_{cp}= the resistance of the concentration polarization layer (m^{-1}); and

R_{tot} = the resistance in total (m^{-1}).

2.4.3 Fouling

Fouling declines the flux when all the operation parameters, such as the pressure, flow rate, temperature, and feed concentration are kept constant (Cheryan 1998).

The simplest mode of fouling is pore plugging. Some particles are small enough to enter the membrane pore structure but can not pass through the membrane, and so physically block it (Russotti and Goklen 2001). At least three kinetic steps are involved in fouling. Firstly, the solute arrives at the membrane surface. Secondly, the solute penetrates into the membrane. Thirdly, the membrane adsorbs the solute (Nilsson 1990). During the membrane fouling, there are two interactions occurring, one is the adsorption in direct relation with the pore structure, the other is a cake layer formation process (Nilsson 1990). Fouling is different from concentration polarization because the materials that caused fouling is deposited on the membrane surface and within the pores. Concentration polarization can be reversed by back flushing the membrane with clean water. However removal of deposited material requires chemical treatment of the membrane to dissolve and flush it away from the membrane. Dilute acid is generally used for this purpose (Su and Colton 1994).

Fouling increases the capital cost because it decreases the flux in the process cycle and therefore more frequent maintenance would be needed. Moreover, cleaning process may require strong cleaning agents which may shorten the lifetime of the membrane (Cheryan 1998).

The factors affecting fouling include the characteristics of a feed stream such as composition, ion strength, hydrophobic interactions, and process engineering factors such as cross flow velocity, pressure and temperature. These characteristics, combined with degree of hydrophobicity of the membrane and its average pore size or MWCO size influnce the membrane fouling rate. The membrane material properties, solute properties and operation parameters and their interactions can be the reasons of membrane fouling (Cheryan 1998).

2.4.4 Operation parameters

2.4.4.1. Transmembrane pressure (TMP)

Both solvent and solute flux increase with increasing TMP. Therefore, it is most efficient to operate a membrane separation system at their lowest TMP that produces an adequate solute flux (Su and Colton 1994).

2.4.4.2 Feed concentration

According to the mass transfer model [Equ (7)], the flux will decrease exponentially with increasing the feed concentration (C_b). This relationship is true regardless of the type of flow or degree of turbulence or the temperature (Cheryan 1998).

2.4.4.3 Crossflow wall shear rate

Wall shear forces increase with increasing average bulk velocity (or flow rate). The higher shear will tend to disperse the solute from the membrane wall and decrease concentration polarization. Increasing the shear rate will increase the cost of energy and pumping. However, protein may be denatured by an excessively high shear rate (Su and Colton 1994).

2.4.4.4 pH and ionic strength

The solution pH will affect the protein sieving through semipermeable ultrafiltration membranes. The protein sieving coefficient has its maximum value near the protein isoeletric point and decreases at pH either above and below the pI (Burns and Zydney 1999).

The surface charge of cells and proteins changes with pH. At the isoelectric point of solute, precipitation may occur and result in increasing the retention of the solute and fouling of the membrane. High salt concentration may cause the protein precipitation, low salt concentration may improve the solubility of some proteins (Su and Colton 1994). The sieving coefficients increase with increasing salt concentration due to the increase in electrostatic shielding at high ionic strength (Menon and Zydney 1999). Pujar and Zydney (1998) also showed that the asymptotic sieving coefficent increased with increasing solution ion strength.

High-performance tangential flow filtration is an emerging technology that enables the separation of proteins of similar size. Reis et al. (1999) have found that the membrane charge produces significant effect on the protein separation and this provides important

insight into the nature of electrostatic interactions controlling transmission of charged proteins through charged membranes.

2.4.5 Development and optimization of a crossflow filtration process

Choosing an appropriate process which includes the membrane and the membrane support is very important for filtration process development and optimization.

Membrane configurations used in CFF can be classified into the following types: plate-and-frame, tubular, hollow fiber, spiral wound, rotary modules and modules designed to create flow through curved channels (Russotti and Goklen 2001).

Filters can be divided to depth filter and screen filter. Depth filters trap the particles when they are pushed into the tortuous paths between the filters. Screen filters consist of a microporous membrane which rejects particles that are larger than the membrane pore size cut off. Screen filters include (Ladisch 2001):

1. Isotropic, symmetric membranes
2. Anisotropic, asymmetric membranes

Microfiltration membranes are typically isotropic, while ultrafiltration membranes are anistropic (Wheelwright 1991). According to their water affinity, these filters can be classified as hydrophilic or hydrophobic filters.

Selection of the proper membrane modules depends on the initial membrane screening studies, cost, space constraints, and ease of use at the final scale. There are many membrane materials commercially available: cellulose acetate, mixed cellulose esters, polysulfone, polyamide, nylon, polytetrafluoroethylene (PTFE), polypropylene, polycarbonate, polyvinylidine-difluoride (PVDF), ceramic and other inorganic

membranes (Russotti and Goklen 2001). When choosing the membrane type, membrane pore size, water affinity, flux, chemical binding, thermal stability, TMP, and flowrate range should be considered.

After a feasible membrane and membrane module are selected, the next step will be the optimization of the process. Usually, we can start from comparing the project's anticipated membrane needs to the membrane manufacturer's recommendation of the operation parameters such as flux, TMP, flowrate and so on. Flux and permeation/retention of products can be further improved by optimization of the membrane operation parameters. These parameters may include feed flowrate, TMP, and backwash frequency.

2.4.6 Enzyme Filtration

Parnham and Davis (1995) found that in the tangential crossflow microfiltration, the flux is relatively insensitive to changes in the transmembrane pressure, but decreases with increasing solids concentration, and increases with increasing shear rate.

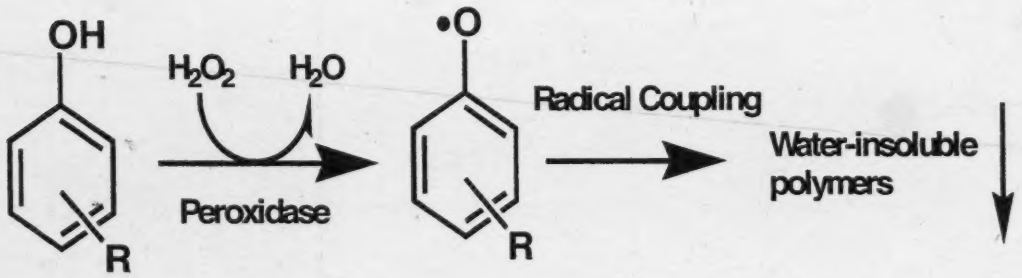
Bowen and Hall (1994) found that for a microfiltration membrane (nominal pore size of 0.2 μm), the filtration rate and transmission of an enzyme (molecular weight of 150,000) depended on the molecular state (degree of aggregation) of the enzyme in solution and on the way in which it interacts with the membrane. Operating at discrete molecules (only at fairly narrow range of pH and ionic strength), the enzyme transmission through the membrane was high (Bowen and Hall 1994). For ultrafiltration, the protein will be concentrated in the retentate if the membrane pore size is smaller than

the enzyme size, so operating beyond the pH and ionic strength at which most enzyme molecules are discrete, may lower the transmission of enzyme through the membrane.

2.5 Enzymatic reaction and reactor

2.5.1 Enzyme catalytic cycle

In the presence of hydrogen peroxide, peroxidase enzymes catalyze the oxidation of phenol and many other aromatics to produce highly reactive free radicals. The free radicals react with each other and polymerize to form eventually water-insoluble polymers. The overall reaction is as follows:



According to the Chance-George mechanism, the whole reaction can be described by the following (Saunders et al. 1964):

$$E + H_2O_2 \longrightarrow E_i + H_2O \qquad (11)$$

$$E_i + AH_2 \longrightarrow E_{ii} + \bullet AH \qquad (12)$$

$$E_{ii} + AH_2 \longrightarrow E + \bullet AH + H_2O \qquad (13)$$

$$\bullet AH + \bullet AH \longrightarrow \text{polyaromatic product} \downarrow \qquad (14)$$

Peroxidases catalyze the oxidation of aromatic compounds by hydrogen peroxide through an oxidation process which involves a cycle of changes in the oxidation state of an iron atom located at the catalytic site of the enzyme (Nicell et al. 1995). The native enzyme undergoes a two-electron oxidation by hydrogen peroxide forming an intermediate state called compound I, which will accept an aromatic compound in its active site and will carry out its one-electron oxidation, liberating a free radical that is released back into the solution, and converting compound I to compound II. A second aromatic compound is accepted into the active site of compound II and is oxidized, resulting in the release of a second free radical and the return of the enzyme to its resting state, completing the catalytic cycle (Adediran and Lambeir 1989; Critchlow and Dunford 1972; Dunford 1991; Nicell et al. 1992).

The iron ion oxidation states change in the cycle as follows (Andersen et al. 1991):

$$Fe^{III} \longrightarrow Fe^{V} \longrightarrow Fe^{IV} \longrightarrow Fe^{III} \qquad (15)$$

As described above, the native peroxidase (ferric, Fe^{III}) undergoes a two-electron oxidation to form compound I (Fe^{V}), integrating one oxygen atom to the ferryl iron ($Fe^{V} = O$) and releasing one molecule of water simultaneously. In the presence of a reducing substrate, Fe^{V} obtains one electron to yield Fe^{IV} and compound II is formed. When another molecule of reducing substrate transfers an electron to compound II, Fe^{IV} is converted to Fe^{III} and returns compound II into the native state (Fe^{III}) (Andersen et al. 1991).

The phenoxy free radicals diffuse from the active center of the enzyme into the solution then react with each other to form dimers, trimers, and so forth. Finally higher oligomers and polymers precipitate and can be easily removed from the wastewater by

coagulation and sedimentation of filtration (Ibrahim et al. 2001; Klibanov et al. 1983; Nicell et al. 1992).

2.5.2 Enzyme inactivation

Peroxidase enzymes are prone to be inactivated during the enzymatic reaction. Klibanov et al. (1983) first suggested that the inactivation of HRP enzyme mostly resulted from the interactions of the phenoxy radicals and the enzyme active center.

However, Nakamoto et al. (1992) have found that the apparent HRP enzyme inactivation resulted mainly from the end-product polymers, which can adsorb the enzyme molecules and inhibit substrate access into the enzyme's active center. Many researchers have studied the addition of protective additives to optimize the enzymatic reactions and improve the cost-effectiveness of the enzymatic treatment method (Ibrahim et al. 1997; Nakamoto and Machida 1992; Nicell et al. 1995; Wu et al. 1993).

Compound III formation in the presence of excess hydrogen peroxide is a reversible inhibition of the enzyme. Nakajima and Yamazaki (1987) presented a compound III formation scheme:

$$E_{ii} + H_2O_2 \longrightarrow (E + O_2^{\cdot -} + H^+) \qquad (16)$$

$$(E + O_2^{\cdot -} + H^+) \longrightarrow E_{iii} + H_2O \qquad (17)$$

Compound II serves as a strong oxidant to cause 1 electron oxidation of H_2O_2 as equation (16). The peroxidase and superoxide radicals then react further to form Compound III as in equation (17).

Compound III is not catalytically active but decomposes spontaneously to native peroxidase (Arnao et al. 1990):

$$E_{iii} \longrightarrow E + O_2^- \quad (18)$$

This is mostly accepted theory of the compound III formation and decomposition though Adediran et al. (1989) thought that compound III is a formal oxidation state and can be reduced to compound I:

$$E_{iii} + e \longrightarrow E_i \quad (19)$$

Irreversible inhibition of the horseradish peroxidase may be caused by the formation of E_{670} (670 refers to the peak wave length in nm at which this compound absorbs light) in the presence of excess hydrogen peroxide (Arnao et al. 1990; Nicell et al. 1992; Villalobos and Buchanan 2002):

$$E_i + H_2O_2 \longrightarrow (E_i \bullet H_2O_2) \quad (20)$$

$$(E_i \bullet H_2O_2) \longrightarrow E_{670} \quad (21)$$

Figure 2-4 shows the full enzymatic reaction cycle of peroxidases.

From the basic Chance-George mechanism given by equations 11 to 14, the stoichiometry between peroxide consumption and aromatic compound removal is 0.5. This does not take into account peroxide consumed in the side reactions shown in Figure 2-4 (formation of compound III and P-670) or the fact that dimers and trimers can take part in the primary catalytic cycle to form the larger polymers which precipitate from solution. Thus, in practice the ratio of peroxide consumed to phenol removed from solution has been reported more frequently to be 1:1 (Nicell et al. 1992; Buchanan and Nicell 1997). Eiii is a temporary inactivation of the enzyme as it slowly reverts to the enzyme's resting state, while P-670 is a permanent inactivation.

In the case of HRP, formation of the permanently inactive enzyme form designated as P-670 has been observed in the presence of excess hydrogen peroxide. Buchanan and

Han (2000) reported the inactivation behavior of ARP to differ from that of HRP in that while ARP was observed to form compound III more quickly in the presence of excess hydrogen peroxide, the formation of P-670 was not observed.

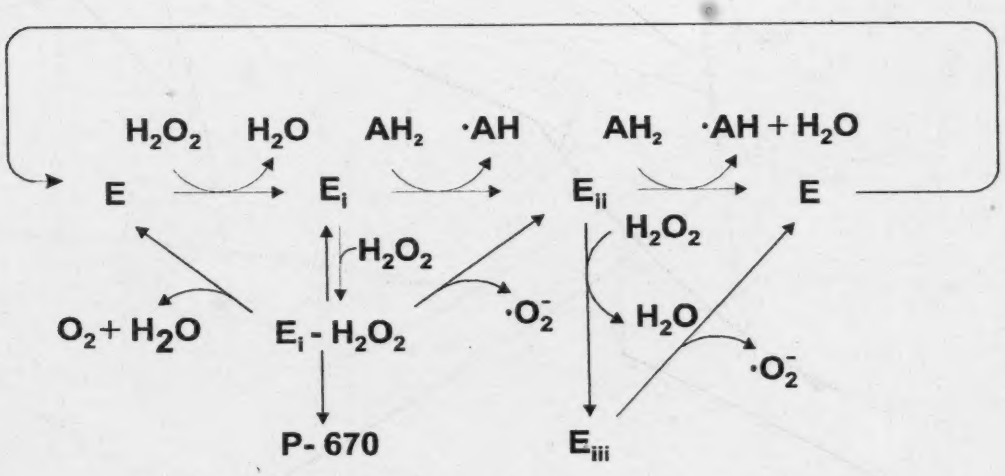


Figure 2-4: The catalytic cycle of HRP with side reactions (after Villalobos and Buchanan 2002).

2.5.3 Enzyme inactivation reduction

To minimize the inactivation of the enzyme, there are three possible approaches: 1) reduce free radical concentration; 2) reduce the amount of enzyme available for inactivation at any instant; 3) use additives to suppress inactivation by end product polymers (Al-Kassim et al. 1994a).

The first two possible approaches can be fulfilled by optimizing the reactor design and were tested by some researchers (Al-Kassim et al. 1994a; Buchanan et al. 1998; Ibrahim et al. 2001; Kennedy et al. 2002; Nicell et al. 1993b; Wu et al. 1999b). The phenol

removal and the lifetime of enzyme can be improved by optimizing the operation conditions such as pH, temperature, retention time, enzyme concentration and phenol/H_2O_2 ratio.

There are different opinions for enzyme addition modes' effects on the phenol removal. Nicell et al. (1993b) reported that maintaining a low enzyme concentration in the reactors can extend the lifetime of the enzyme. Moreover, single and multiple continuous stirred tank reactor (CSTR) performances were superior to batch reactor performance even at a lower retention time. Al-kassim et al. (1994a) reported that when either hydrogen peroxide or *coprinus macrorhizus* peroxidase (CMP) or both was added in discrete aliquots over 0.5 hour, the phenol removal efficiency can be improved significantly. Wu et al. (1999b) also determined that the step addition of hydrogen peroxide increased the phenol removal efficiency by HRP. However, Ibrahim et al. (2001) reported that the step addition of the hydrogen peroxide did not reduce the ARP requirement and did not improve the removal efficiency significantly when ARP was used to remove phenol from refinery wastewater. The possible reasons for the above difference are the type of peroxidase, type of wastewater and style of the reactors they used in the experiments.

Using additives to suppress the inactivation of peroxidases also has been tested by many researchers and determined to be effective in most situations (Buchanan and Nicell 1998; Ibrahim et al. 2001; Kinsley and Nicell 2000; Nakamoto and Machida 1992; Nicell et al. 1995; Wu et al. 1993; Wu et al. 1999a; Wu et al. 1999b). Nakamoto and Machida (1992) studied that the use of proteins, such as gelatin and polyethylene glycol (PEG-1000) to suppress the inactivation of the enzyme during the reaction and the amount of

the peroxidase required reduced to 1/200 when using the above additives. Kinsley and Nicell (2000) have found that only the PEGs with molocular weight more than 1500 can improve the lifetime of enzyme and the degree of improvement increased with increasing molecular weight. Wu et al. (1993) developed an empirical model to determine the minimum PEG concentration required for at least 95% phenol removal:

$$(PEG)_{min} = 0.0065 + 0.0241\ C_{ph} \qquad (22)$$

where $(PEG)_{min}$ = the minimum PEG dose ($g\ L^{-1}$) under the minimum dose of HRP ($U\ mL^{-1}$) for at least 95% phenol removal

C_{ph} = the concentration of phenol (mM)

Nicell et al. (1995) also determined the minimum PEG dose as the following:

$$[PEG] = 19.2\ [AH_2]_0 \qquad (23)$$

Where [PEG] = the minimum amount of PEG ($mg\ mL^{-1}$) for maximum protection of HRP; and

$[AH_2]_0$ = the initial concentration of phenol (mM)

Ibrahim (1997) determined the PEG relationship with the initial phenol concentration when using ARP:

$$PEG_{min} = 20 + 10\ C_{ph} \qquad (24)$$

Where PEG_{min} = the optimum PEG dose for 1, 3, 5, 7 and 9 mM phenol concentration

C_{ph} = the initial phenol concentration (mM)

Ibrahim et al (1997) observed that in some cases the excess of PEG has a negative effect on the phenol removal efficiency when using ARP. Their possible explanations were that the PEG affected the coagulation by alum negatively and increased the solubility of the byproducts.

2.5.4 Enzyme reaction factors

2.5.4.1 Hydraulic Retention Time (HRT)

HRT is an important factor for technical and economical reasons. The HRT will decide the reactor's size that is related to the capital cost. Buchanan et al. (1998) have determined that for a low hydraulic retention time, a continuous flow stirred tank reactor (CFSTR) needed much more enzyme than a plug flow reactor (PFR), however, for longer hydraulic retention time, a multi-stage CFSTR used the enzyme more efficiently than a PFR.

Optimization of the reaction time under different enzyme and hydrogen peroxide concentrations and other conditions such as pH, temperature will balance the operation cost and capital cost and then achieve the best economical efficiency (Wu et al. 1993).

Kennedy et al. (2002) have found that at 22 °C and pH 7.2, complete removal of 100 mg L^{-1} 2,4-dichlorophenol (DCP) needed approximately 1 min by 1 unit mL^{-1} SBP in a batch reactor. The reaction is so fast that the HRT in the enzymatic-based reactors will be much shorter than conventional biological reactors even if some phenolic compounds need longer reaction time than 2,4-DCP needs.

Al-Kassim et al. (1994b) reported that at the optimal of pH 8.0, HRP removed 95% 1 mmol L^{-1} phenol at 0.8 U mL^{-1}, but 5 hours were needed; CMP was shown to remove 1 mmol L^{-1} phenol at 0.6 U mL^{-1} to removal 90% phenol in 10 minutes, and an additional 5% removal needed over 100 minutes.

Usually, 1 to 3 hour reaction time has been adopted to test the phenolic compounds removal in the enzyme-base reactors.

2.5.4.2 Enzyme concentration

The rate and extent of an aromatic or phenolic compound removal from the wastewater is a function of enzyme concentration. Some researchers have established the enzyme consumption models from different views.

Wu et al. (1993) determined the minimum HRP dose required to achieve 95% removal in the presence of PEG at phenol concentrations from 1 to 10 mM. The mathematical equation is:

$$(HRP)_{min} = 0.0435 + 0.0048\, C_{ph} + 0.0031\, C_{ph}^{2} \qquad (25)$$

where $(HRP)_{min}$ = the minimum HRP dose (U mL^{-1}) required to achieve at least 95% removal, and

C_{ph} = the concentration of phenol (mM).

Nicell et al. (1995) established the enzyme dose needed for different degrees of removal (from 0.3 to 0.9) and it revealed a strong linear relationship:

$$E = C\,[AH_2]_0 \qquad (26)$$

Where E = enzyme dose (U mL^{-1}) required to achieve a specific removal with or without additives;

C = the enzyme dose coefficient (U mL^{-1} mM^{-1}) with or without additives;

$[AH_2]_0$ = the initial phenol concentration (mM)

$C = aX^{b}$

X = the desired conversion of phenol

Without PEG, a = 1.478, b = 0.989;

With PEG, a = 0.0698, b = 1.358

Kennedy et al. (2002) related the SBP requirement to pH for 100 mg L^{-1} 2,4-DCP removal:

99% removal:

$$SBP_{min} = -0.0173\ pH^3 + 0.352\ pH^2 - 2.3838\ pH + 5.54 \quad (27)$$

And 50% removal:

$$SBP_{min} = -0.0045\ pH^3 + 0.0879\ pH^2 - 0.5713\ pH + 1.2719 \quad (28)$$

Where SBP_{min} = minimum SBP required, U mL^{-1}

pH = pH of the solution

Ibrahim et al. (1997) also determined that the ARP minimum dose for 95% removal:

$$E_{min} = 0.548\ C_{ph} \quad (29)$$

Where E_{min} = the minimum dose of ARP (U/ml) required to achieve more than 95% phenol removal

C_{ph} = the phenol concentration (mM)

From these models and experiences, higher enzyme concentration can achieve higher removal of phenolic compounds. Also, higher phenolic compounds concentration need higher enzyme dose. However, higher enzyme concentration will definitely increase the operation cost.

2.5.4.3 pH

Like other proteins, enzymes possess many ionizable groups so that pH may affect the conformation of the enzyme, the binding of the substrate, the catalytic activity of the groups in the active site of the enzyme. The pH also affects the stability of the enzyme.

Therefore, the optimal operational pH is often a balance between the effects on enzyme activity and enzyme stability (Wiseman 1985).

Klibanov et al. (1980) and Klibanov (1982) studied the effect of pH on aromatic removal using HRP and identified the optimal process pH to be neutral or slightly acidic.

Nicell et al. (1993) determined that the optimum pH for phenol is 8.1 when using HRP. However, Wu et al. (1993) reported that the optimum pH was 8.5 without PEG and the optimum pH was 8.0 with PEG.

Nakamoto et al. (1992) reported that phenol removal efficiency was more than 90% in the range of pH 5-8, but the efficiency would decrease rapidly when pH exceeded 9 when the initial phenol concentration was 10 g L^{-1} and HRP 10 mg L^{-1}.

The Optimum pH for CMP was determined to be from 7 to 9 for different aromatic compounds (Al-Kassim et al. 1994b).

The optimum pH for SBP treatment of 2,4-DCP was around 8.2 and removal declined rapidly that higher pH values in the presence of low SBP concentrations (Kennedy et al. 2002).

Like other peroxidases, CIP has its own optimum pH range when treating the aromatic wastewater. The optimum pH range is from pH 7- 9. Ikehata and Buchanan (2002b) determined that the optimum pH was 7.2 using the crude CIP. Kauffmann et al. (1999) reported that the optimum pH was around 8 using CIP, similar with HRP. Although at pH 7.5, enzyme activity is the maximum, the CIP optimum pH for phenol removal was 9.0 (Masuda et al. 2001b).

For phenol removal by enzymatic method, neutral or slightly alkaline conditions seem good for most of the aromatic compounds.

2.5.4.4 Temperature

Like other chemical reactions, the rate of enzyme catalyzed reactions increases with the increasing temperature, and can be described by Arrhenius relationship (Wiseman 1985):

$$k = A' e^{-\frac{E_a}{R'T}} \quad (30)$$

Where k = the reaction rate constant;

A'= the Arrhenius constant;

E_a = the energy of activation;

R' = the gas law constant; and

T = the absolute temperature.

The higher temperature can increase the enzyme activity but decrease the enzyme stability (Wiseman 1985). As a result, there is a balance point for enzyme to achieve the highest reaction rate.

The reaction rate catalyzed by SBP is significantly slower at lower temperature. It increased by a factor of about 20 as the temperature was increased from 4 °C to 22 °C (Kennedy et al. 2002).

Masuda et al. (2001b) observed that the relative CIP activity reached the peak at 40 °C and then decreased rapidly, the phenol removal reached the maximum at 0 °C. The possible explanation is the low temperature can lower the inactivation of the enzyme by reducing the rate of free radicals generation.

2.5.4.5 Hydrogen peroxide

As discussed in Section 2.4.2, in the real situation, the hydrogen peroxide to phenol ratio is around 1 because of compound III formation and because several repetitions of the catalytic cycle are needed to grow the product polymer to the point where it will precipitate from solution. Peroxide to phenol ratios greater than the optimum stoichiometry will cause more severe inactivation of the enzyme and then decrease the phenol removal because excess hydrogen peroxide can produce E_{iii} and possibly P-670.

One-to-one stoichiometry was found for phenol, 2-, 3-, and 4-chlorophenol, 2,4-dichlorophenol, and 2-, 3-, and 4- methylphenol (Nicell et al. 1992).

Wagner et al. (2001) determined that the hydrogen peroxide to phenol molar ratios were 1.14 and 1.24 respectively for different samples of a petroleum refinery wastewater.

Wu et al. (1993) reported that the peroxide to phenol molar ratio was 1.0 –1.1 and that the chemical additives did not affect the stoichiometry.

CIP also has similar stoichiometry because it has similar characteristics with HRP. Masuda et al. (2001b) reported that at different CIP concentrations, the phenol removal reached the maximum at different stoichiometry. The discrepancy might result from the enzyme preparation or the polymerization and precipitation mechanisms. They concluded that the ratio of hydrogen peroxide to phenol as (n-1)/n, where n is the degree of polymerization. This is in agreement with the scheme suggested by Nicell (1992)

Therefore, the ratio of hydrogen peroxide and phenol between 1.05 and 1.1 is good for the removal of aromatic compounds.

2.5.4.6 Reactor configuration

Better reactor configuration can lower the overall cost. In the enzyme-base reactors, the phenolic compound concentration is the most important parameter for the design. The phenolic compound concentration determines the minimum enzyme requirement and the optimum hydrogen peroxide dose. The enzyme dose will significantly affect HRT and therefore affect the reactor's size. Another important parameter is the flowrate.

Wu et al. (1999b) recommended a plug-flow system for treating the phenolic wastewater regardless of the phenol concentrations because the plug-flow reactor performance is similar to the batch reactor and can use step addition of hydrogen peroxide or enzyme. Usually, the higher the flow rate, the better to use a plug-flow system.

A plug-flow reactor required shorter retention time for equal phenol removal. Conversely, a multi-stage CFSTR would use enzyme more efficiently at longer HRT because the enzyme inactivation rate was lower (Buchanan et al. 1998).

Nicell et al. (1993b) also reported that the multiple stage of CSTR could extend the lifetime of the enzyme beyond the single CSTR for the same retention time.

2.5.4.7 Other factors

Other factors, such as impurities in crude enzyme, wastewater constituents and coagulant, can affect the phenol removal.

The phenol removal efficiency has been reported to decrease with increasing the CIP purity. The medium component, such as polypepton or yeast extract, protein or amino acids in the crude enzyme solution improved the phenol removal efficiency because these

impurities can suppress the inactivation of CIP, furthermore, more acidic and higher molecular weight proteins were more effective in suppressing the inactivation of CIP (Masuda et al. 2001a).

The reducing substrate other than phenols such as sulfite and thiosulfate required more hydrogen peroxide because they competed with the enzymatic phenol oxidation for the available peroxide. On the other hand, the oxidation substrate such as sulfite and sulfide enhanced the phenol conversion. Certain wastewater components like cyanide and Mn(II) have negative effects on the enzymatic reaction (Wagner and Nicell 2002).

Cationic polymer coagulants containing amino groups such as hexamethy-lenediamine epichlorohydrin polycondensate and polyethy-leneimine improved the phenol removal efficiency by preventing the inactivation of the peroxidase due to reaction products adsorption (Tatsumi et al. 1994).

2.5.5 Reaction products

Yu et al. (1994) indicated that reaction conditions, which had relatively high phenol and peroxide concentrations in the presence of low enzyme concentration, would tend to favor the formation of large amount of dimmers, rather than of larger polymers. If the concentration of these dimmers exceeded their solubility limits, precipitation would occur and result in a lower that normal stoichiometry being observed.

If the polymers fail to precipitate, they may be further oxidized through the catalytic action of HRP resulting in the formation of higher molecular polymers with even lower solubilities (Nicell et al., 1993). We can examine the product precipitation character and optimize the operation to improve the product precipitation property.

There is little information about how to treat the precipitate from the enzyme-based treatment process. It deserves much attention about the fate of the solids produced in the treatment process because we are not sure how the polymer will affect the sludge treatment and its effect on the soil and groundwater.

Heck et al. (1992) examined the toxicity of reaction products from enzymatic oxidation of several phenolic pollutants and concluded that some phenolic pollutants will increase acute toxicity as a result of enzymatic oxidation. Ghioureliotis and Nicell (2000) revealed that both SBP and HRP did not detoxify aqueous phenol and substituted phenol solutions to levels that were consistent with the low residual quantity of parent substrate following treatment. The enzyme-catalyzed polymerization process may be more advantageous when attempting to detoxify wastewaters that contain multiple phenolic contaminants. Alternatively, when only one contaminant is predominant in the wastewater, it may be advantageous to add a non-toxic and inexpensive co-substrate to enhance treatment (Ghioureliotis and Nicell 2000).

3. Materials and methods

3.1 Materials

Coprinus cinereus UAMH 4103 was obtained from the University of Alberta Microfungus Collection and Herbarium, Alberta, Canada. ACS grade solid phenol (MW 94.11 g mol^{-1}, purity 99% or greater), ACS grade hydrogen peroxide (30% w/v), 98% 4-aminoantipyrine (4-AAP), ACS grade potassium ferricyanide, ACS grade sodium bicarbonate, and ACS grade monobasic and dibasic (anhydrous) sodium phosphate, potassium ferricyanide, sodium hydroxide, sulfuric acid were purchased from Fisher Scientific Canada (Edmonton, Alberta, Canada). Glucose assay kit was bought from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Enzyme assay solutions were buffered to pH 7.4 using a mono- basic-dibasic sodium phosphate buffer prepared according to the method of Gomori (1955). PDA, Dextrose, Peptone, Yeast extract, Malt extract manufactured by Difco were purchased from Fisher Scientific Canada. PEG-3500 and alum were purchased from Fisher Scientific Canada. Ultrapure water produced by Elgastat Maxima Water Purification System (Elga Ltd., High Wycombe, Bucks, England) was used for all reagent preparation. Extra pure oxygen from Praxair (Edmonton, Alberta, Canada), was used for supplying oxygen in the fermentor.

Purified CIP (Reinheitzahl number (RZ; $Abs._{404\ nm}/Abs._{280\ nm}$) = 1.9), prepared by ion exchange chromatography with a DEAE cellulose (DE52) column and size exclusion chromatography with a Sephadex G 100 column, in 0.1 M phosphate buffer was kindly provided by Keisuke Ikehata, a Ph.D. candidate at the University of Alberta.

3.2 Equipment

A Hewlett-Packard HP 8453 UV-Visible Spectrophotometer was used for the measurement of UV and visible spectra of samples, and colorimetric assasys for the activity of peroxidase and concentration of hydrogen peroxide and phenol.

An Ultrospec 2000 UV-Visible spectrophotometer from pharmacia Biotech Ltd. (Cambridge, England) was used for glucose assay. Glass and quartz semi-micro cuvettes with a 1 cm optical path and a 1.5 ml volume (Hellma Ltd., Canada) were used in all spectrophotometric measurements.

An Immersion Model Cc1 (Thermo Haake, Paramus, New Jersy) in combination with a polypropylene water bath was used for glucose assay.

A Centra-GP8R Refrigerated Centrifuge (International Equipment Company, Needham Heights, Massachusetts) was used for solid-liquid separation of samples.

A Bioflo 110 Fermentor (figure 3-1) from New Brunzwick Company (Edison, New Jersey, USA) was used to culture *Coprinus cinereus UAMH 4103* and produce peroxidase.

A crossflow filtration module (borrowed from Dr. Smith) was used for the ultrafiltration membrane holder. A 5KH36KN90 vacuum pump (from Fisher Scientific) was used for the vacuum filtration. Coarse filtration, 0.45 μm, 0.22 μm membrane were bought from Fisher Scientific Company and used in the vacuum filtration system, 100 kDa 10 kDa membrane were bought from Pall life science Company (Mississauga, Ontario, Canada) and used in the crossflow ultrafiltration system.

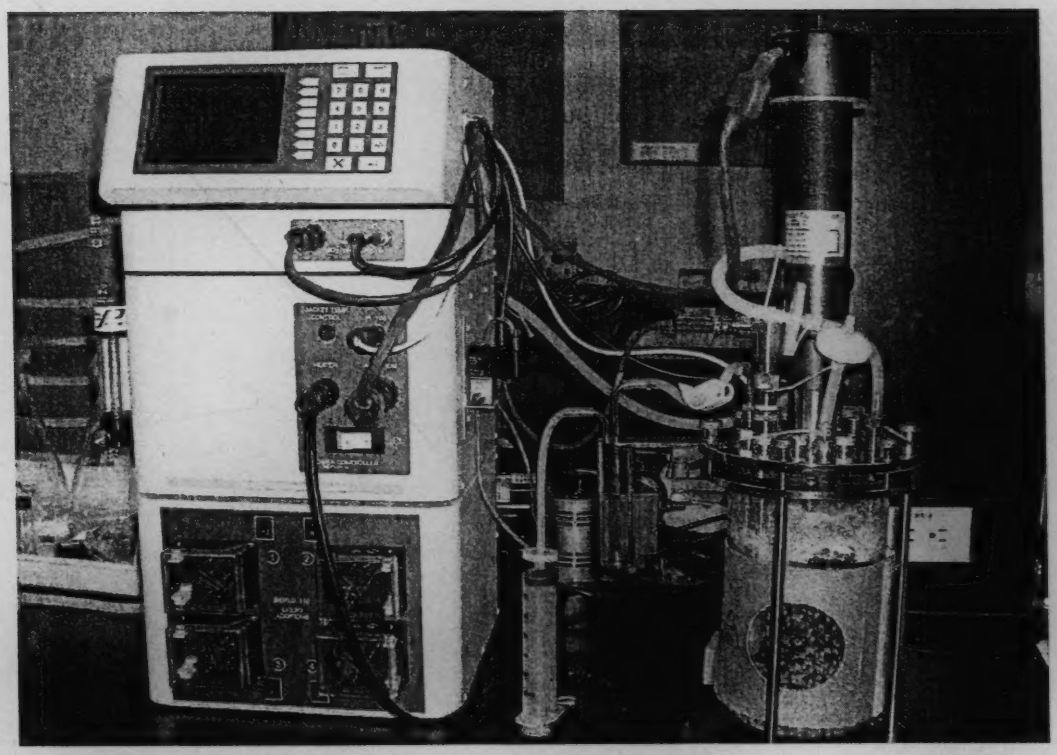


Figure 3-1 Bioflo 110 Fermentor.

A Prep/Scale spiral wound filter module (Figure 3-2) was bought from Millipore Company (Bedford, USA). The membrane material is polysulfone. The molecular weight cut-off size is 10 KDa. The membrane area is 0.23 m^2.

A 25-feet Teflon tubing with 1.27×10^{-2} m inner diameter was purchased from Fisher Scientific Canada (Edmonton, Alberta, Canada). It was used to construct a 7.62 m plug-flow reactor. At 1 m, 2 m, 4 m and the end, the sampling ports were set (Figure 3-3).

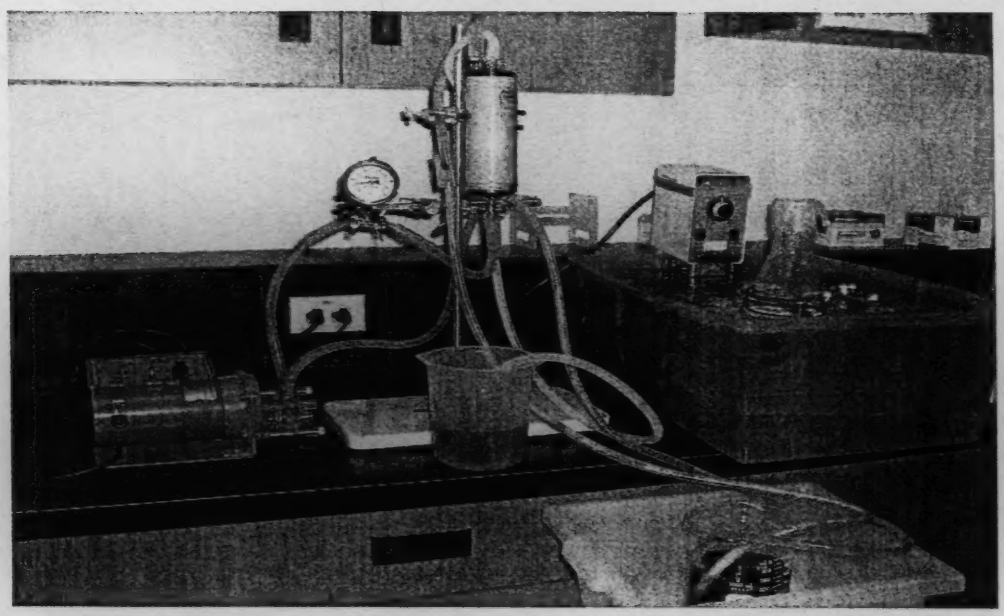


Figure 3-2: Prep/Scale-TFF system.

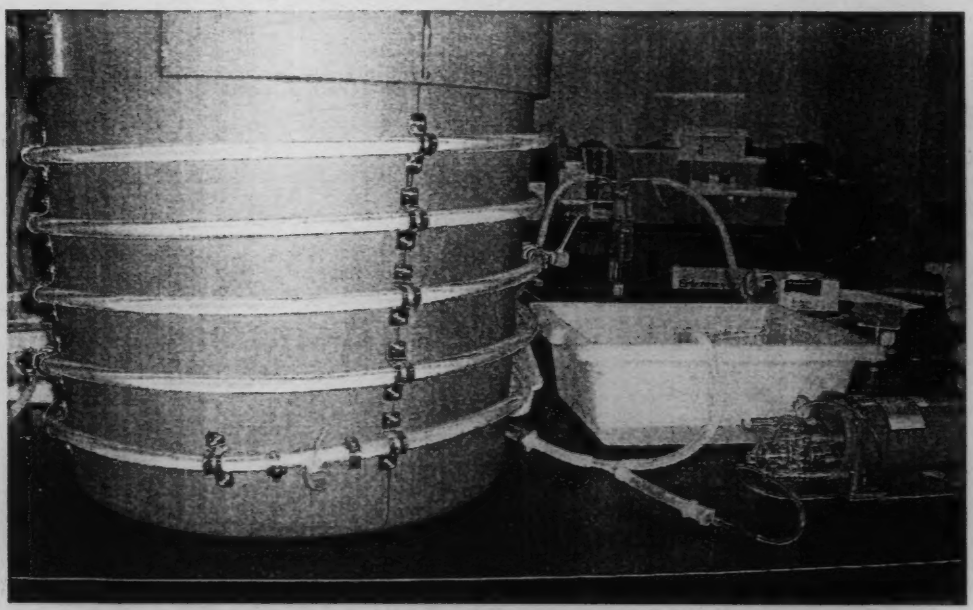


Figure 3-3: Plug-flow reactor.

Several different size peristaltic pumps were used to pump the fermentation broth to the Prep/Scale filter module and to add phenol solution and enzyme solution/hydrogen peroxide solution into the plug-flow reactor.

Several magnetic stirrers were used to stir the batch reactors.

3.3 Methods

Enzyme activity assay

The phenol-AAP colourimetric assay described in Buchanan and Nicell (1997) was used to determine enzyme activity.

Phenol determination

Phenol concentrations were measured by a colorimetric assay described in Buchanan and Nicell (1997).

Hydrogen peroxide determination

Hydrogen peroxide concentrations were determined by the end point assay described in Buchanan and Nicell (1997).

Glucose determination

Glucose concentrations were determined by a colorimetric assay (Sigma Manual). 1.5 mL assay volume was composed of 0.5 mL sample and 1.0 mL glucose reagent. At time 0, the assay reagents were transfer the test tube in a water bath (37°C). After exactly 30 min, 1ml 12N H_2SO_4 was added to stop the reaction. The absorbance was measured at 540 nm in a spectrophotometer and calculate the glucose concentration using the standard curve.

COD determination

The standard closed Reflux method 5220 D (American Public Health Association 1995) was used to determine COD value.

Culturing method

Coprinus cinereus UAMH 4103 was cultivated in the PDA plate at 25°C for one to two weeks. Approximately 0.1cm portion of surface mycelia from the seed culture were inoculated into 250 ml Erlenmeyer flasks which contained 100 ml of a liquid medium (medium A) composed of 1% dextrose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract and cultivated on a rotary shaker at 175 rpm and 25°C. After 6 or 7 days, the biomass was transfered into the fermentor. The fermentor was operated at 25°C, 50-100 rpm agitation and 30% dissolved oxygen until the dextrose was consumed and the peroxidase reached the maximum.

Another medium (medium B) composed of 2% dextrose, 0.5% peptone, 0.3% yeast was tried by the same procedure. The fermentor was operated at 30°C, the agitation and the dissolved oxygen were the same as the above.

Membrane separation method

The crude peroxidase solution was filtered by a vacuum filtration system at room temperature and pH 7-8. The vacuum pressure was at 69 to 103 kPa. The coarse filter paper was used first, then 0.45 μm and 0.22 μm microfiltration membranes were used in series to remove the biomass and big particles.

After 0.22 μm membrane filtration, the peroxidase solution was filtered by 100 kDa and 10 kDa ultrafiltion in a Plate-and-Frame module or in a 10 kDa Prep/Scale spiral wound module at room temperature and at pH 7-8. The feed pressure was set to 206.79-

345 kPa depending on the fouling of the membrane. For the spiral wound module, the retentate was returned to the crude enzyme feed solution container, after the liquid volume has decreased to 100 to 200 ml, a certain amount of pure water was added and the process was continued. Several additions of pure water are needed until the permeate looks clean.

Batch reactor

Both the initial phenol and hydrogen peroxide concentration were 1mM, a known volume of crude enzyme or filtered enzyme were added into different reactors (the final concentration of the enzyme was 1 U mL^{-1} to start the reaction). The magnetic stirrers were used to mix the solution completely. After 3 hr reaction, mixing was stopped and10 ml liquid were sampled from each reactor, transferred to centrifuge tubes and then centrifuged at 3000 × g for half an hour.

Plug-flow reactor

All the experiments were carried out at room temperature. The hydraulic retention time was set as one hour for all experiments. The reaction mixture was unbuffered and sodium hydroxide and sulfate acid were used to adjust pH value of the reaction solution. Control samples were used in each set of assays, which measured the actual concentrations of phenol and H_2O_2. All assays were performed in duplicate using the same stock reactants. The reactions were initiated by adding crude CIP or hydrogen peroxide to the plug flow reactor which containing the phenol, PEG and H_2O_2 or crude CIP. Samples were withdrawn from 1m, 2m, 4m and the end sampling ports of the plug-flow reactor after two hydraulic retention times.

When the addition of enzyme or hydrogen peroxide was made at 0 m and 4 m ports, the samples were withdrawn from 1 m, 2 m, and the end ports of the plug-flow reactor after two hydraulic retention times.

200 μL catalase (1mL = 1 mg) was added into all the samples right after the samples were withdrawn from the reactor to stop the reaction. Alum (final concentration of 0.2 g L^{-1}) was added to start the coagulation and flocculation by shaking the sample tube for five minutes and then using centrifuge to settle the polymers at 3000 × g for 30 minutes.

Figure 3-4 shows the whole integrated enzymatic treatment system process flow.

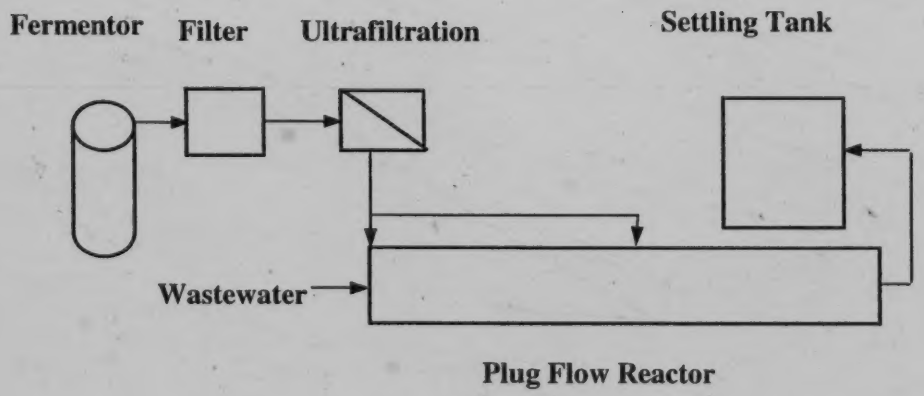


Figure 3-4: The whole integrate enzymatic treatment system.

4. Results and Discussion

4.1 Production of CIP

4.1.1 Results

CIP production was tested under 2 operating conditions and with 2 media.

During the first several preliminary runs, only compressed air was used as an oxygen source. At the end of each run, dissolved oxygen reached 0 and could not satisfy the need of the fungus although the agitation speed was 100 rpm. The maximum peroxidase activity achieved in these several runs was 17.35 U mL^{-1}. After that, at the beginning of a run, compressed air was used as the oxygen source until the fungus biomass increased to the point that compressed air could not satisfy the need of the fungus, it was then replaced with extra dry pure oxygen. This modification improved the production of enzyme. As shown in Figure 4.1-1, the peak enzyme activity was almost 20 U mL^{-1}. Choi et al. (2002) also found that a high oxygen source was required to produce lignin peroxidase and manganese peroxidase.

After these preliminary runs using a fermentor during which valuable operation experiences was obtained, production of CIP with the peak enzyme activity of 19.2 U mL^{-1} was achieved by *C. cinereus* at day 7 (see Figure 4.1-1). At the same time, the glucose concentration decreased to almost 0. Figure 4.1-1 shows the enzyme activity increased with time, and after day 7, when the enzyme activity reached the peak, the enzyme activity decreased with time. A plausible explanation for the enzyme disappearing would be the reuse of the material in metabolism because the enzyme disappearance often occurrs when there is a demand for nitrogen-containing 'building blocks' for metabolism if nitrogen starvation occurs (Thurston 1972). During the

production period, the pH value was not controlled, and fluctuated as shown in Figure 4.1-1.

Sequencing-batch production was tried for three periods (see Figure 4.1-2). From Figure 4.1-2, after 2 to 3 days acclimation in the new environment, *C. cinereus* fungi started to secret the enzyme. After the third day, the glucose was used rapidly and the enzyme concentration increased quickly. At the seventh day, the glucose concentration reached 0. Almost one day later, the enzyme concentration reached its maximum. At the peak enzyme production, pH was 7.2.

In preparation for the second batch, most of the liquid and some fungi suspected to be dead were taken out from the fermentor. New media were added to the fermentor to yield a final total volume of 2L as in the previous trial. All these operations were carried out in a biosafty hood to minimize the possibility of contamination. During this sequence, no acclimation time was needed for the fungi. The fungi started to secrete the enzyme as soon as the media were added. At the fourth day after the addition of new media, the glucose was consumed, and one day later, the enzyme concentration reached its maximum. The pH fluctuation was similar to that observed during the first trial (See Figure 4.1-2).

A third batch was attempted after the second, but because of problems with the stirrer, the fungi died two days after the addition of the new media and the enzyme activity started to decrease at this time (see Figure 4.1-2).

Figures 4.1-1 and 4.1-2 reflect the enzyme production, glucose consumption, and pH fluctuation with time when a broth containing 1% dextrose, 0.5% peptone, 0.3% yeast, 0.3% malt was used. For the economic consideration, batch tests using flasks were

conducted to determine whether malt was needed. All other conditions were kept the same as in previous trials. The fungi were grown at 25°C in shaker flasks set at 175 rpm. As shown in Figure 4.1-3, some difference in enzyme production was observed. However, the difference was so small that it was determined to be not statistically significant at 5% level. It seems that malt is not necessary for CIP production. Thus, it was deleted from the experiments that followed. The time course of the enzyme production in the shaker flasks was longer than that in the fermentor. A possible reason is that DO limitation may have occurred because the shaker flasks were not aerated.

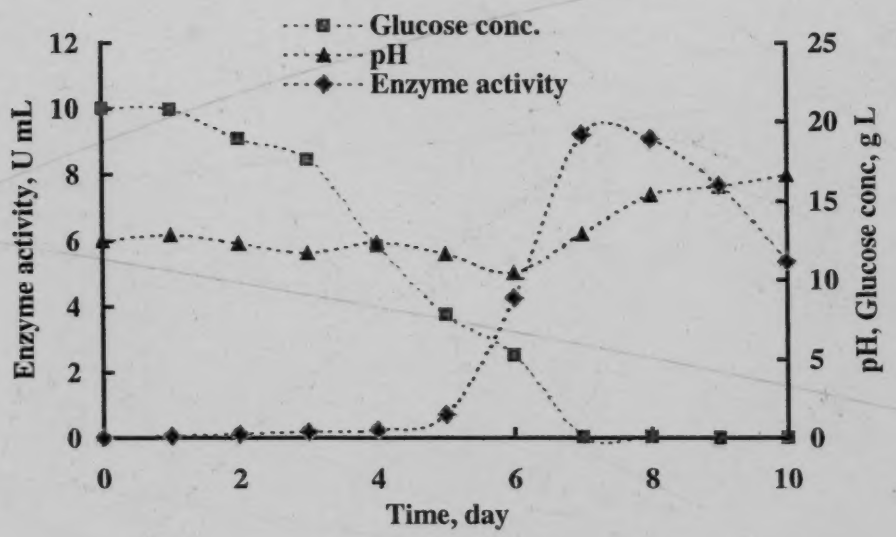


Figure 4.1-1. Glucose concentration, Enzyme activity and pH variation during growth of *C. cinereus* in batch fermentor, Temperature=25°C, DO set at 30%, Stirrer speed at 50 –100 rpm

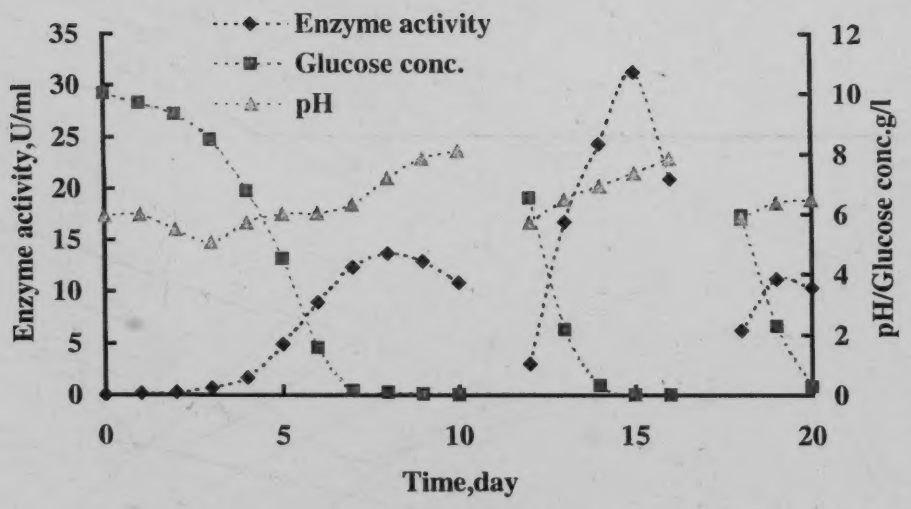


Figure 4.1-2 Semi-batch production of CIP in the fermentor, Temperature=25°C, DO set at 30%, Stirrer speed at 50 –100 rpm

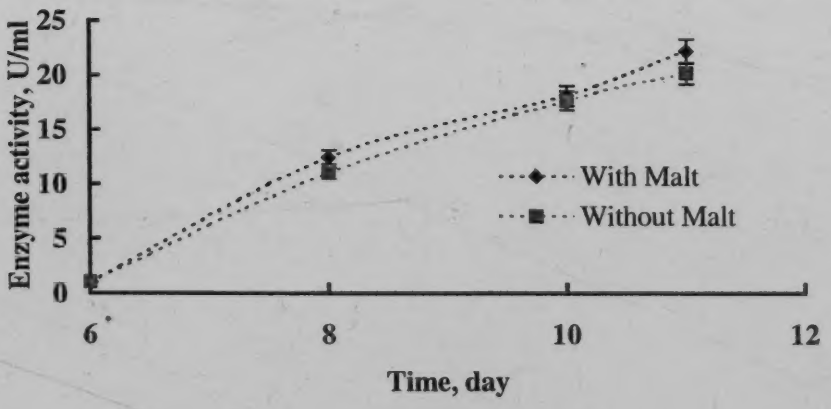


Figure 4.1-3: Enzyme production in shaker flasks in a media with malt and without malt, Temperature=25°C, shaking speed at 175 rpm.

The enzyme production at a different condition was tried. The medium (medium B) contained 2% dextrose, 0.5% peptone and 0.3% yeast. The operation temperature was fixed at 30°C. The fermentor agitation speed started at 50 rpm and was then increased to 100 rpm when fungi grew to a certain amount in the fermentor. At the ninth day, the glucose was consumed and one day later the enzyme concentration reached the maximum of almost 60 U mL^{-1} (see Figure 4.1-4). This was much higher than the previous runs. The peak was delayed two days compared with the previous culturing media and operation conditions. The plausible reason for this is the latter media contained more dextrose and the fungi used the dextrose for longer period time.

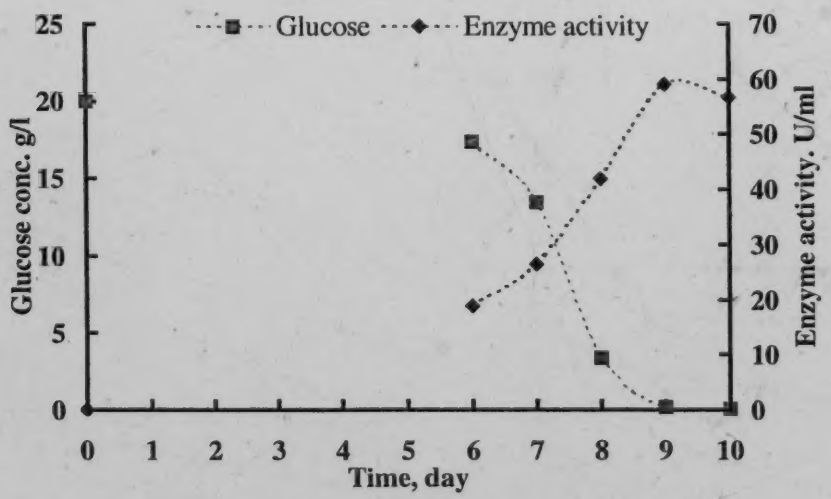


Figure 4.1-4: Production of enzyme in fermentor at Temperature=30°C, DO set at 30%, Stirrer speed at 50 –100 rpm

4.1.2 Discussion

Coprinus cinereus UAMH 4103 was used in the batch and sequencing-batch fermentation. It is known that the growth of this fungus can be affected by the glucose concentration, dissolved oxygen, pH and temperature among other culturing conditions. For economy of energy, the temperature of the fermentor was maintained at 25°C at first (near room temperature). Although the control of pH may be extremely important when optimizing the enzyme productivity (Stanbury et al. 1995), the medium pH was not adjusted or buffered and allowed to change naturally because this project just tested the feasibility of low cost production of CIP peroxidase. The dissolved oxygen became a key factor in these experiments. The dissolved oxygen in the fermentor depends on two factors: oxygen supply, which decides how much oxygen is added into the fermentor, and agitation, which decides how the oxygen is distributed and how much the oxygen was consumed in the fermentor.

The agitation speed is another very important factor. Rapid agitation promotes oxygen mass transfer. However, the basidiomycetes are known to be sensitive to shear stress in the liquid culture, and the peroxidase production can be affected greatly by the agitation rate (Tsujimura et al. 1994). Therefore, strong agitation can not be used. After several trials, the following procedure was determined: at the beginning of the run, the amount of fungi was less, the agitation speed was set at 50 rpm. After the fungus grew and appeared to fill the fermentor, the agitation speed was changed to 100 rpm. Sometimes, the agitation motor did not work well due to a faulty control system. If the agitation speed increased rapidly to more than 100 rpm but less than 300 rpm, the agitation produced observable negative effects on the growth of the fungus and therefore decreased the

production of the enzyme. When the agitation rose unexpectedly higher than 300 rpm, it caused more serious problems. The severity of the problem depended on how fast the agitator rotated, how long the problem lasted and how often the problem happened. The most serious situation resulted in the whole fungal mass being killed. So the agitation system (bearing, impeller gland, motor and automatic controller) is very important in the fermentation process, especially for CIP production by the fermentor.

Another factor was the humidity of the compressed air and the pure oxygen. Before the gas is introduced into the fermentor, it should pass through pure water first, or the exhaust gas will bring out some water from the fermentor and the liquid media in the fermertor will become less and less.

A comparison of the production at two different media and operation conditions shows that the use of medium B yielded almost three times more CIP than that of medium A. Medium B contained twice as much dextrose as did medium A but it did not contain malt. Figure 4.1-3 shows that the omission of malt did not inhibit the production of CIP. The possible reason is that the concentration of the dextrose affected the growth of the fungus, therefore the secretion of CIP by fungi increased a lot. Longer growth time in medium B may include longer stationary phase during the growth cycle compared with medium A, and therefore, CIP can be secreted by fungi for a relatively longer time and more CIP can be produced. This should be further studied to verify the medium composition effects on the production of CIP by *coprinus cinereus* UAMH 4103 strain.

Also there was another factor, temperature, to affect the production of enzyme. In medium A, 25 °C was used and in medium B, 30 °C was used. As we know, higher

temperature can increase the growth of fungi before the limit. At higher temperature, fungi should be active and then secrete more enzyme.

4.2 Membrane separation system

The harvested culture medium supernatant was passed through coarse filtration paper (Q8) to remove large biomass. Then 0.45 μm and 0.22 μm microfiltration membrane were used in series to remove small particles. The enzyme activity was not affected by the removal of these kinds of particles (Figure 4.2-1)

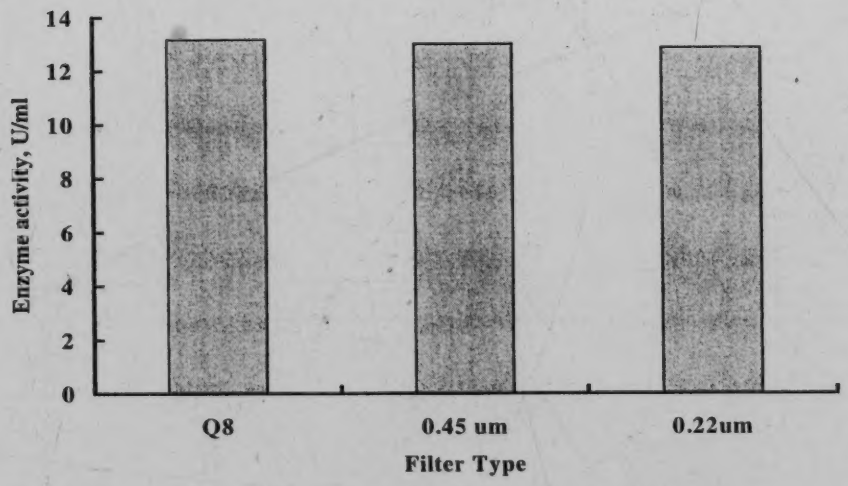


Figure 4.2-1. Enzyme activity after vacuum filtration through different membranes, at room temperature, pH 7-8, vacuum pressure at 68.93 to 103.4 kPa (10-15 psi).

The COD of the enzyme mixture was measured before and after each of these filtration steps. No significant difference in COD was observed. The filtrate from the 0.22 μm membrane was then subject to ultrafiltration in a crossflow filtration system (Tangential Flow Filtration system).

In this plate-and-frame filtration system, 100 kDa and 10 kDa ultrafiltration membranes were tested. As shown in Figures 4.2-2 and 4.2-3, neither enzyme concentration nor COD reduction was achieved with the 100 kDa membrane. It was not used in further experiments.

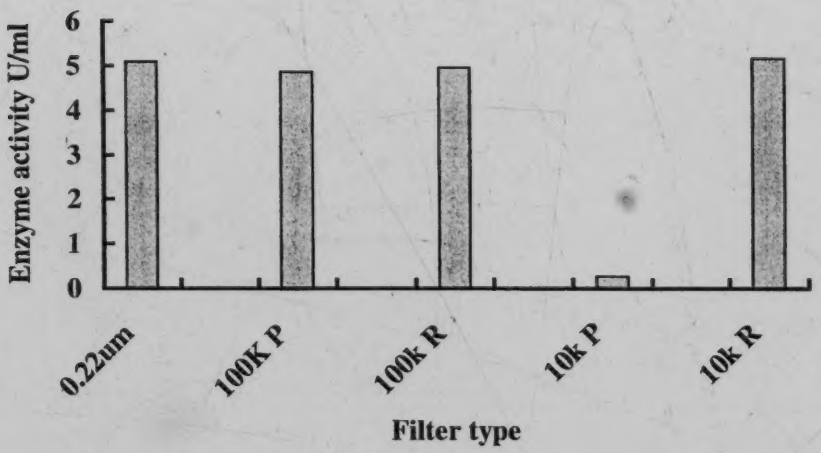


Figure 4.2-2. Enzyme activity in permeate and retentate of various membrane.

0.22 μm membrane operated at room temperature, pH 7-8, vacuum pressure at 68.93 to 103.4 k Pa. 100 kDa and 10 kDa membrane operated at room temperature. pH 7-8 and the pressure at 206.79-344.65 kPa.

Note: 100 k P --- permeate of 100 kDa membrane

100 k R---retentate of 100 kDa membrane

10 k P and 10 k R has the same meaning as 100 kDa

It appeared that the 10 kDa membrane could retain almost all of enzyme, as a result, the enzyme activity in the permeate was negligible. However in the retentate, the enzyme

activity did not markedly increase. No difference in enzyme concentration among the rententate solutions of the 10 kDa, 100 kDa and 0.22 μm membrane was observed. Although the enzyme activity in the permeate solution was almost 0, the enzyme activity in the retentate solution did not increase as expected.

From a mass balance analysis of the trials using the 10 kDa membrane (see Table 4.2-1), 37.1% of the enzyme was lost in the filtration system even though the membrane used in this study was the lowest protein-binding according to Pall Life Science company. The main reasons for the mass loss are: 1) the membrane area was so small that the membrane was fouled very fast; 2) the plate-and frame module was not designed for protein separation and it adsorbed/captured a lot of enzyme. These were verified when the module was opened and a lot of colored material was observed on the surface of the membrane and in the module.

Table 4.2-1 Mass balance of enzyme in plate-and-frame module test

	V, ml	Enzyme activity, U/ml	Enzyme mass, U	Enzyme parts, %
Orig	100	6.68	668.25	--
Retentate	37	10.58	391.35	58.6
Permeate	63	0.46	28.87	4.3
Mass loss	--	--	248.02	37.1

Note: The test was operated using a 10 kDa membrane at 40 Psi and pH 7.0 until the retentate flow rate was almost none.

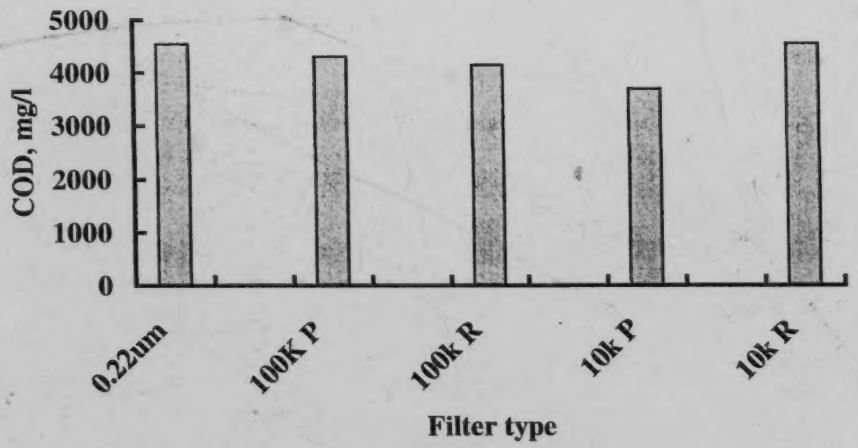


Figure 4.2-3. COD value in permeate and retentate of various membrane

Note: The meaning of 100 k P, 100 k R, 10 k P and 10 k R have the same meaning as Figure 4.2-3.

The operation conditions are the same as Figure 4.2-2.

The plate-and-frame separation system did not work well for COD removal. From Figure 4.2-3, it can be observed that the COD of filtrate and retentate after treatement with different membranes were very similar. It shows enzyme did not contribute a lot of COD, but the macromolecules did. There may be other secreted products of the fungus, the leftovers of peptone, yeast and malt and their reaction products or other things. 100 kDa membrane and bigger than 100 kDa membrane can not separate them from the harvested media. 10 kDa membrane could retain almost all of the enzyme, but it could not produce big effects in COD removal.

Because the plate-and-frame module system can not efficiently concentrate the enzyme solution, a Prep/Scale spiral wound module was bought for this purpose.

The Prep/Scale spiral wound module proved very effective at concentrating the enzyme solution (See Table 4.2-2). When the enzyme solution volume was decreased to 19.3%, the enzyme concentration was multiplied 4.48 times in the retentate solution. The recovery was 86.6% and the activity loss was only 13.4%. The activity loss comprised the enzyme loss in the system and the permeate solution, although enzyme activity in the permeate solution was not detectable.

The COD removal in the Prep/Scale module system was very effective. When the feed solution was just 100 to 200 ml, 1000 ml pure water was added into the feed solution beaker, at the same time the ultrafiltration was continuing. 500 ml pure solution was added into the feed solution beaker at similar situation, thus, some COD were washed out from the concentrated enzyme solution, COD in the concentrated enzyme solution was decreased. Finally the COD was removed up to 83.3%. This is beneficial for the phenolic wastewater treatment system because it will not increase the organic pollutants burden very much.

Table 4.2-2: Enzyme activity and COD in Original, retentate and permeate solution in Prep/Scale module test

	V, ml	Enzyme activity, U/ml	Enzyme Unit, U	Recovery, %	Mass COD,mg	COD parts, %
Original	1500	14.75	22129	--	7683	--
Retentate	290	66.08	19164	86.6	1283	16.7
Mass loss	--	--	2965	13.4	--	--
COD removal	--	--	--	--	6400	83.3

Note: the test was operated at 68.95 kPa (10 Psi) and pH 7.0. The enzyme solution was pretreated by Q8 and 0.22 μm filtration.

4.3 Phenol removal using batch reactors

The different filtrate and retentate (10 kDa permeate not used) were used to remove phenol with hydrogen peroxide without PEG addition. The phenol removal efficiency after 3 hours reaction is shown in Figure 4.3-1. The removal efficiencies were all more than 85%. In the batch reactors, both the initial phenol and hydrogen peroxide concentration were 1 mM, a known volume of crude enzyme or filtered enzyme were added into different reactors (the final concentration of the enzyme was 1 U mL^{-1}) to start the reaction.

This demonstrated that the enzyme produced by *Coprinus cinereus* UAMH 4103 in a batch fermentor could remove the phenol well (Figure 4.3-1 and 2).

The comparison experiments of the phenol removal by ultrafiltrated (10 kDa MWCO) and purified CIP were carried out at pH 7.0 with different reaction conditions shown in Figure 4.3-2. Figures 4.3-1 and 4.3-2 indicate that the purity of enzyme solution does not affect the phenol removal efficiency. Conversely, in the buffered reactor, the impurities can improve the phenol removal.

Some interesting results are shown in Figure 4.3-2. If phosphate buffer was not used in the batch reactor, the ultrafiltered and purified enzyme can remove almost the same amount of phenol. If PEG was added into the unbuffered reactors, the phenol removal was not improved. This indicates that if the reaction solution is not buffered, ultrafiltrated and purified CIP can achieve almost the same phenol removal efficiency and PEG can not help to improve the phenol removal efficiency.

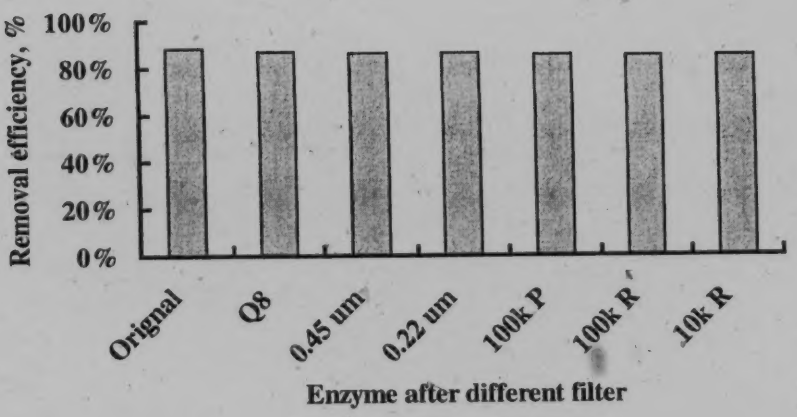


Figure 4.3-1 Phenol removal efficiency at different filtrate and retentate, at room temperature, medium magnetic stir ($[enzyme]_0$=1 Unit/ml, $[H_2O_2]_0$ =1 mM, $[phenol]_0$ =1 mM)

In the reactors that were buffered by 0.1 M pH 7.0 phosphate buffer, the results were different. Figure 4.3-2 shows that the purified enzyme removed the least phenol if the solution was buffered and PEG was not used. In the same situation, ultrafiltrated enzyme can achieve higher phenol removal efficiency (more than two times). As shown in Figure 4.3-2, there was no significant difference in the phenol removal achieved by enzyme that had been purified by ultrafiltration and that achieved the enzyme that had been purified further (see Materials and Methods). Similarly, no significant difference in phenol removal by the two enzyme preparations was observed in the buffered solution to which 200 mg PEG /L had been added. This removal was also not statistically different from that observed in the unbuffered solution. However, in the where buffered solution was treated in the absence of PEG, phenol removal by both enzyme preparations was

suppressed, relative to the unbuffered case. Nevertheless, the less pure (ultrafiltered) enzyme removed approximately twice the phenol that the purified enzyme did under these conditions. This suggests that the phosphate buffer inhibited the enzymatic phenolic reactions, and that PEG or the natural impurities that remained in the ultrafiltered enzyme solution suppressed the inhibition effectively.

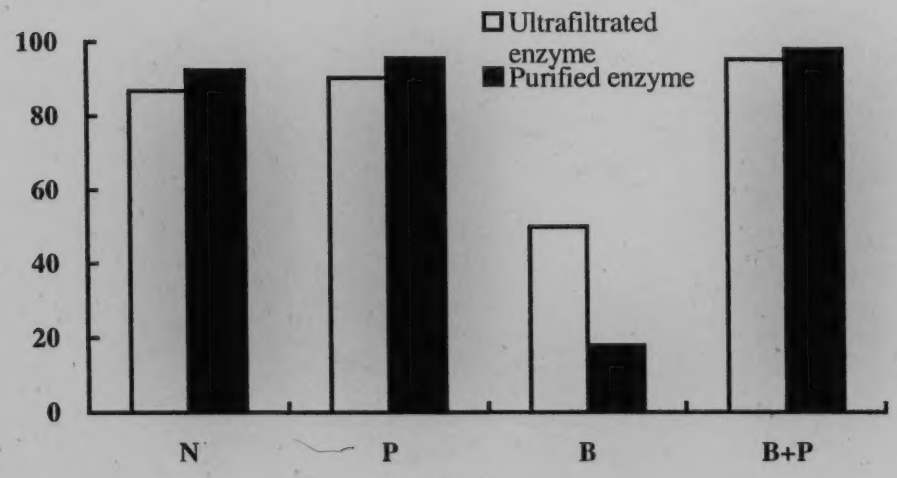


Figure 4.3-2: Comparison of the phenol removal by ultrafiltrated and pure enzyme at pH 7, $[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, $[CIP]_0$ = 0.5 U mL^{-1}, error bars represent 95% confidence intervals.

Note: N: no buffer or PEG, final pH=7.57;
P: 0.2 g L^{-1} PEG, final pH=7.45
B: phosphate buffer (pH 7.0);
B+P: phosphate buffer (pH 7.0) and 0.2 g L^{-1} PEG
RZ of pure enzyme is 1.9 in pH7 phosphate buffer (0.1 M)

As discussed in the introduction, some researchers, like Alberti (1982), Dec (1994), Flock (1999), and Masuda (2001a), have obtained similar results that the crude

peroxidase can achieve the same or ever better phenol removal from the wastewater than pure peroxidase. However, there is no comparison between buffered and unbuffered aromatic wastewater. There are some comparisons between addition of protective additives and no addition of protective additives in buffered or unbuffered aromatic wastewater.

Cooper et al. (1996) determined that in the presence of PEG, phenol removal from the synthetic wastewater, which used pH 7.4 phosphate buffer, was greater than from the foundry wastewater, which was the real wastewater but also buffered by phosphate buffer solution, using the same HRP dose. PEG additives improved the removal efficiency significantly. These results are similar with that in the buffered solution, which is that PEG additive can significantly improve the phenol removal in the buffered solutions.

Ibrahim et al. (1997) used hydrochloric acid or sodium hydroxide to adjust pH value of the reactants and determined that the best results could be achieved by dividing the treatment scheme as: phenol removal by enzymatic reaction and followed by conventional coagulation and sedimentation process to remove the soluble reaction products. At optimized situation pH 7, ARP dose of 0.6 U mL^{-1}, PEG dose of 30 mg L^{-1}, hydrogen peroxide dose of 1.1 mM and alum dose of 25 mg L^{-1}, the phenol removal was 94%. PEG additives improved the phenol removal efficiency by 28 to 70 % for 1 and 10 mM phenol solution, respectively. The possible reason for the difference between Ibrahim's results and those of the present study is that type and purity of the peroxidase, alum dose, and pH are different.

4.4 Phenol removal in plug-flow reactors

Based on the findings in the batch reactors, no buffer was used in the plug-flow reactor. All reactions used 10 kDa ultrafiltrated CIP enzyme. This will increase the cost efficiency for the whole enzymatic treatment system.

4.4.1 Hydraulic retention time (HRT)

All of the trials with the plug-flow reactor show that most of the phenol removal would occur in the first 20 minutes after which phenol removal increased more slowly with time. Al-Kassim et al. (1994b) reported that 90% phenol removal was achieved at optimal conditions in the first 10 minutes and an additional 5% removal needed 100 minutes at 0.6 U mL^{-1} CMP, completely removing phenol from the wastewater in 5 hours. The phenol removal shown in Figures 4.4-1 to 4.4-7 does not stop at the last point (60 minutes) although one hour retention time was set for all plug-flow experiments. The reasons for setting one hour as the retention time are to: 1) shorten the experiment time in a reasonable range, which can reflect the enzymatic phenolic reaction; 2) leave some space to improve the future design. As shown in the Figures 4.4-1 to 4.4-7, extending the hydraulic retention time to more than one hour can improve the phenol removal efficiency.

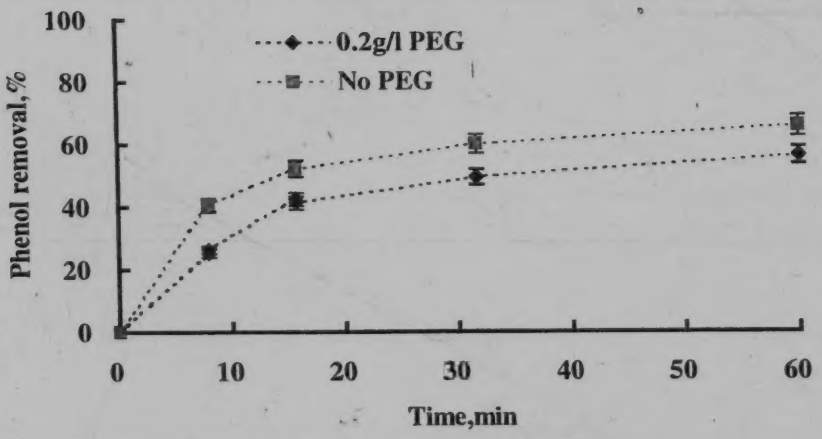


Figure 4.4-1: Time courses of phenol removal at pH7.0, $[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, $[CIP]_0$ = 0.65 U mL^{-1}, $[PEG]_0$ = 200 mg L^{-1}

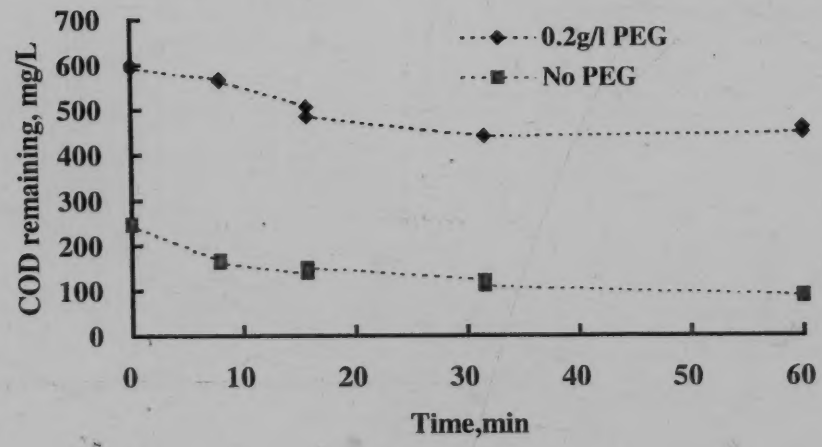


Figure 4.4-2: Time courses of COD Removal at pH7.0, $[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, $[CIP]_0$ = 0.65 U mL^{-1}, $[PEG]_0$ = 200 mg L^{-1}

4.4.2 Effect of PEG additives

Although some chemical and protein additives can improve the phenol removal efficiency by HRP, ARP and SBP in the buffered reactions(Ibrahim et al. 1997; Kinsley and Nicell 2000; Nakamoto and Machida 1992; Wu et al. 1993), PEG can not improve the phenol removal efficiency when using crude CIP at pH 7.0 (see Figures 4.4-1, 3, 4). Conversely, PEG additives produced some negative effects on the phenol removal in these experiments.

In Figure 4.4-1, the phenol removal without PEG was almost 10% higher than that with PEG from the second sample point to the end. In Figure 4.4-3, the difference in phenol removal in the presence or absence of PEG gradually increased to around 10% removal at the end when step addition of CIP was used.

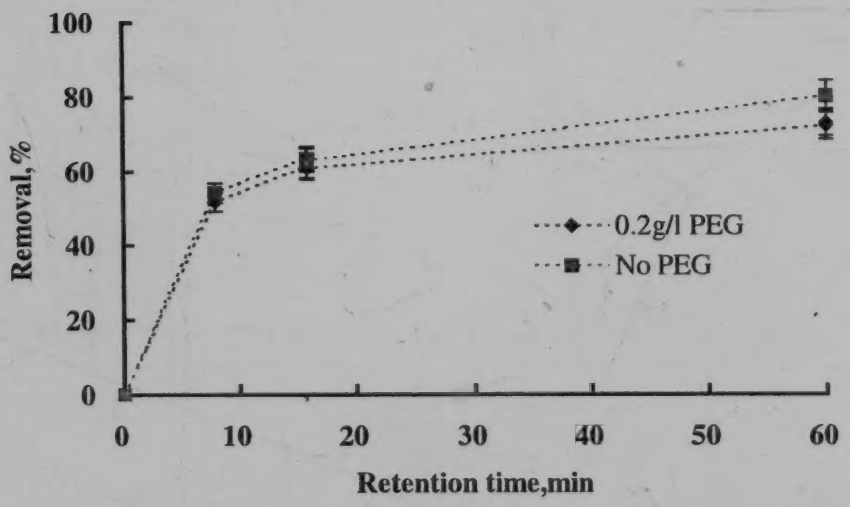


Figure 4.4-3: Phenol removal at pH7.0, $[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, [total CIP] = 0.65 U mL^{-1}, 0m 0.45 U mL^{-1}&4m 0.2 U mL^{-1}enzyme

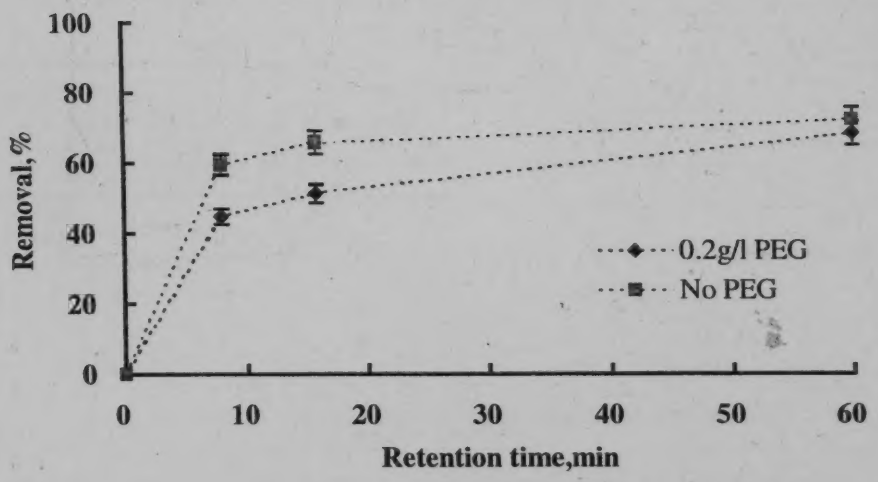


Figure 4.4-4: Phenol removal at pH7.0, $[Phenol]_0 = 1$ mM, $[CIP]_0 = 0.65$ U mL^{-1}, [total H_2O_2] = 1.1 mM, 0 & 4m 0.8 & 0.3mM H_2O_2.

In Figure 4.4-4, the difference started to expand from the beginning, reached the maximum around the second sample point, then approached zero at the end, when step addition of hydrogen peroxide was used. This indicates that as more ultrafiltrated CIP was added in the reactor, the effect of PEG diminished.

Buchanan et al. (1998) reported that the observable effect of staged HRP addition in the presence of PEG would be to reduce the rate of phenol removal slightly.

Ibrahim et al. (1997) also observed similar results when using ARP in their experiments. They believed that the presence of PEG in the mixture might have a negative effect on the coagulation by alum or it might affect the solubility characteristics of the byproducts.

In these experiments, alum was added to the samples to coagulate the aromatic polymer, thus it should not produce a significant effect on the phenol polymerization.

One possible reason is the PEG might change the solubility characteristics of the byproducts, therefore decrease the phenol removal efficiency. Another possible explanation is that the impurities in the crude enzyme and PEG can affect each other or the impurities and PEG worked together to produce the similar effect on phenol removal as chitosan, therefore decrease the phenol removal efficiency as observed in Figure 4.4-1, 3, 4. Wagner et al. (2001) found that chitosan had an adverse effect on phenol removal in concentrations exceeding 80 mg/l and PEG had a maximum protection at 5 mg/l with no further improvement at higher PEG doses when they used HRP to treat a petroleum refinery wastewater.

Figure 4.4-2 shows that the PEG addition increased chemical oxygen demand (COD. Comparing the two curves in Figure 4.4-2, PEG was not removed from the wastewater once it was added. The COD difference between the PEG curve and no PEG curve was not significant statistically at 5% level. In the step addition experiments (step addition of CIP or hydrogen peroxide), the same results were obtained (data not shown).

In this case, PEG can not improve the phenol removal efficiency and only increases the organic load in the effluent. Therefore, not adding PEG will improve the cost-effectiveness of the enzymatic treatment system when using crude CIP at pH 7.0.

4.4.3 Effect of CIP concentration

As shown in Figure 4.4-5, phenol removal is a function of CIP concentration. Phenol removal efficiency can increase with increasing CIP concentration in the reactor. It was the most significant at the first sample point. If 1.5 U mL^{-1} CIP was used, almost 90% phenol can be removed in the first 20 minutes, but later the trend of phenol removal was

much slower. When 0.65 U mL^{-1} CIP was used, the phenol removal was around 65% at 60 minutes which is approximately 25% less than that removed by 1.5 U mL^{-1} CIP. However, the removal trend was steeper than that by 1.5 U mL^{-1} CIP after the second sample point.

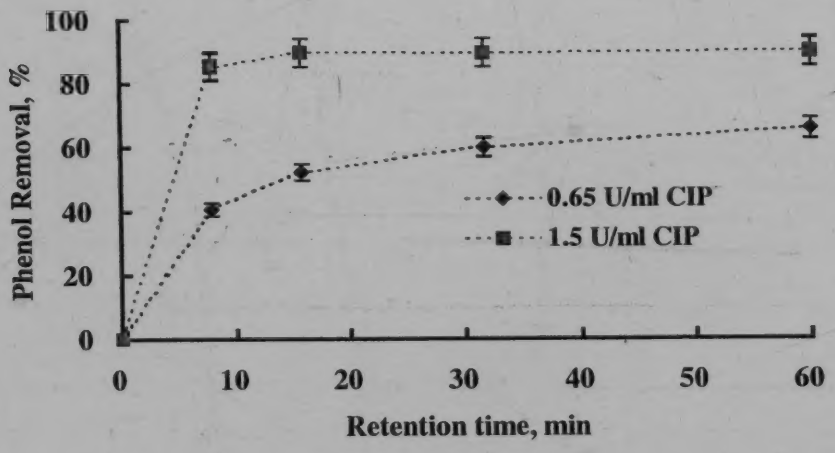


Figure 4.4-5: Phenol removal with different CIP concentration at pH7.0,

$[H_2O_2]_0 = 1.1$ mM, $[Phenol]_0 = 1$ mM

Figure 4.4-5 shows that higher enzyme concentration can increase the reaction rate, however, higher enzyme concentration also can increase the inactivation rate. At around 20 minutes, phenol removal almost stopped when 1.5 U mL^{-1} enzyme solution was used. In contrast, phenol removal continued to go on when 0.65 U mL^{-1} enzyme solution was used and extended until one HRT or more. This suggested that the lower enzyme concentration in the reactor will extend the lifetime of the enzyme, the higher enzyme concentration in the reactor will increase the inactivation rate.

Higher enzyme concentration shortened the reaction time and therefore would shorten the required hydraulic retention time. If field space is very tight and cheaper enzyme is available, higher enzyme concentration may be adopted to save the reactor size. It is

certain that the balance of reactor size and enzyme cost should be considered when designing the enzymatic treatment system.

4.4.4 Step addition of CIP

The effect of step addition of CIP is shown in Figure 4.4-6. There were two addition points: one at 0 m and the other at 4 m. The total CIP amount added into the plug-flow reactor was 0.65 U mL^{-1} in every trial. The control curve is the phenol removal efficiency when 0.65 U mL^{-1} CIP was added at 0 m point (Addition method A). As shown in Figure 4.4-6, when the addition amounts of CIP at 0 m and 4 m were equal to 0.33 U mL^{-1} (Addition method B), the phenol removal was lower than that of addition method A at the first 37 minutes, after that crossed the phenol removal curve of the addition method A when another 0.33 U mL^{-1} was added at 4 m addition port and kept higher than the phenol removal of addition method A. Also, the phenol removal by addition method B was faster than that by addition method A after about 10 minutes. This indicates that the step addition of enzyme in the reactor can suppress the inactivation of the enzyme by reducing the chance of the interactions between the hydrogen peroxide and the enzyme intermediates and the interactions between the enzyme and phenoxy radicals.

When 0.45 U mL^{-1} CIP was added at the reactor inlet and 0.20 U mL^{-1} at the 4 m mark (addition method C), the phenol removal was always higher than those of addition method A or B. According to Figure 2-4 (The catalytic cycle of HRP), in the main cycle, one molar enzyme will catalyzed 2 molar phenols, however, there are side reactions, so the phenol removal rate will depend on the balance of phenol removal reaction rate and the side reaction rate (inactivation rate of enzyme). Buchanan et al. (1998) pointed out

that reducing the amount of active enzyme would reduce not only the rate of compound III formation but also the maximum possible phenol removal rate. Therefore, the phenol removal increasing by step addition of CIP depends on the balance of these two actions.

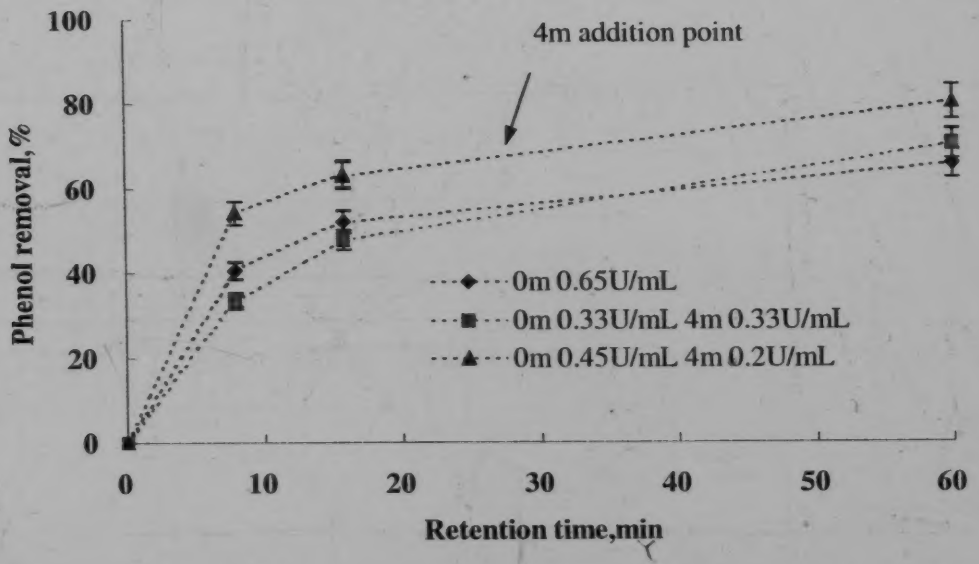


Figure 4.4-6: Phenol removal with step addition of CIP at pH7.0, $[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, [total CIP] = 0.65 U mL^{-1}

For addition method B, before the second addition of enzyme, the phenol removal abided by the trend that a higher concentration enzyme increases the phenol removal reaction. However, higher concentration enzyme (Method A) also increases the rate of inactivation, therefore after around 10 minutes, the reaction rate looks lower than that by addition method B. After the second addition of CIP, the available enzyme concentration was higher by addition method B than that by addition method A because in addition method A more enzyme was inactivated.

For addition method C, the phenol removal was higher than those by the other addition methods. The likely explanations are the inactivation of enzyme in addition method C

was lower than that in addition method A because the enzyme concentration was lower, and the phenol removal reaction rate was higher in the addition method C than that in addition method B.

Al-Kassim et al. (1994a) believed that the extended availability of hydrogen peroxide and peroxidase activity improved phenol removal in a discontinuous semibatch reactor. Different step addition methods will affect the phenol removal differently. Addition method C is better than addition method B at a fixed total CIP amounts. So the appropriate step addition of enzyme can suppress the inactivation of enzyme (the side reaction) and can use the enzyme more efficiently requiring the use of less enzyme. In practice the optimal mode of reagent addition would need to be determined experimentally for the waste stream in question.

Another possible explanation is that the plug-flow reactor was not ideal, and the mixing of the reagents was not consistent. Wu et al. (1999b) suggested a mixing tank before the plug-flow reactor can reduce HRP inactivation and consequently improve the enzyme turnover. Ibrahim et al. (1997) also used a mixing tank before the plug-flow reactor and a flocculation tank was used before the sedimentation tank to help improve the mixing and remove phenol at the same time.

4.4.5 Step addition of hydrogen peroxide

The effect of step addition of hydrogen peroxide is shown in Figure 4.4-7. Similar to the effect of step addition of enzyme, step addition of hydrogen peroxide improved the phenol removal efficiency. At the beginning, step addition of hydrogen peroxide can remove about 20% more of the phenol than the one point addition at the reactor inlet.

Before the second addition of hydrogen peroxide, the difference of phenol removal between these two addition modes almost kept the same. After the second addition, these two curves approached together slowly. From the trend, the two curves may almost merge. The step addition of hydrogen peroxide may suppress the inactivation by the interactions of hydrogen peroxide and the enzyme intermediates (Baynton et al. 1993; Hinter et al. 1996). The inactivation rate was lower when step addition of hydrogen peroxide was used, therefore, phenol removal reaction rate was faster than that of one point addition.

It is known that the optimal hydrogen peroxide and phenol molar ratio is 1.1. If this molar ratio is less than 1.1, the phenol removal can not complete, if more than 1.1, it will cause the inactivation of enzyme and then decrease the phenol removal efficiency (Masuda et al. 2001b; Nicell et al. 1992; Wu et al. 1993). In these experiments, the molar ratio of total amoung of hydrogen peroxide added to initial phenol was 1.1.

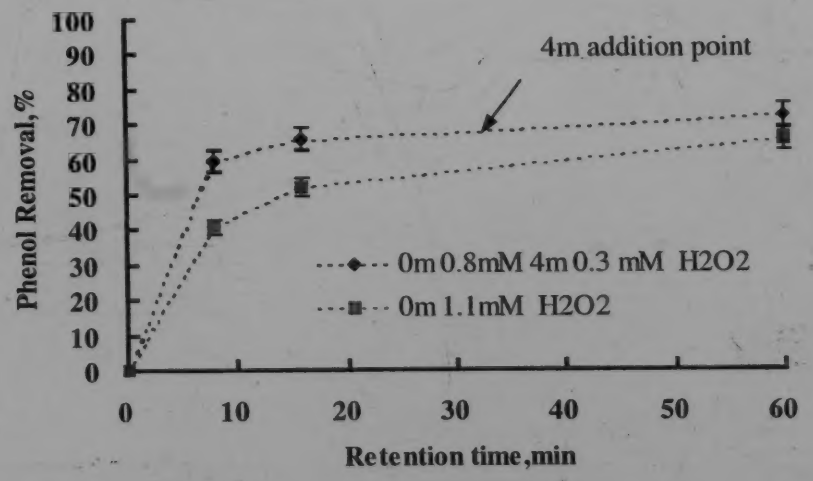


Figure 4.4-7: Phenol removal with step addition of H_2O_2 at pH7.0, $[Phenol]_0 = 1$ mM, $[CIP]_0 = 0.65$ U mL^{-1}, $[total\ H_2O_2] = 1.1$ mM.

5. Conclusion

5.1 Production of CIP

The feasibility of enzyme production by a stirred tank fermentor has been examined. It was proved that using the stirred tank fermentor to produce CIP is possible. Not only higher glucose concentration can produce more *coprinus cinereus* peroxidase, but also higher temperature can also increase the production rate of *coprinus cinereus* peroxidase.

5.2 Purification of enzyme

It was shown that the ultrafiltrated CIP can remove the same phenol as purified CIP. The purification process includes biomass removal by fiber filters, macromolecular removal by 0.22 μm membranes and enzyme concentrating by 10 kDa ultrafiltraion. The system not only can concentrate the enzyme concentration, but also can remove more than 80 % of COD in the crude enzyme solution.

5.3 Plug-flow reactor

All reactions in the plug-flow reactor were not buffered by any buffer solution because the batch tests had already shown that it is not necessary to use buffer in the reagents when ultrafiltrated CIP is used. The pH was adjusted by sodium carbonate and sulfuric acid.

Higher enzyme concentration can achieve higher phenol removal efficiency and shorten the reaction time. A balance of the enzyme concentration and the reactor size should be considered to minimize the capital cost and operation cost.

Step addition of both enzyme and hydrogen peroxide can improve the phenol removal efficiency although step addition of enzyme was more efficient than step addition of hydrogen peroxide. These are useful for the design of the plug-flow reactor in the real situations.

PEG was proved to be unnecessary in the plug-flow reactor when using crude enzyme at pH 7.0. Batch tests also showed that PEG would not improve the phenol removal efficiency significantly when no buffer was used in the reagents. Therefore, PEG additives may not be necessary in the real situation.

6. Recommendation

6.1 Production of CIP

Since the second batch production of CIP during the sequencing-batch production, the production cycle can be shortened because fungi do not need the acclimation period any more. If the glucose concentration is kept as a certain level, fungi will continuously secret CIP. Therefore, it is better to further study the CIP production using sequencing-batch, semi-batch production mode or continuous production mode. It has special meaning for the practical application.

If possible, the optimization of the fermentor operation may be attempted. The optimization may consider the following factors: What kind of media can produce the most enzyme and least COD? What level of glucose concentration maintained during the continuous production can maximize the production of enzyme? What pH, agitaion speed, oxygen supply, and temperature should be maintained in the fermentor to maximize the production of enzyme and keep economy?

If possible, a growth and enzyme production model can be established.

6.2 Purification of CIP

Optimizing the operation conditions should be studied in the future.

The optimization conditions may include the flux and the retention flowrate, transmembrane pressure drop, pH, ionic strength and backwashing. Also how to minimize the fouling and maximize the protein recovery are very important in the future studies.

6.3 Reactor design and operation optimization

In the future studies, optimizing the plug-flow reactor design should be considered firstly because the ideal plug-flow reactor has the same phenol removal as the batch test, which had a better phenol removal.

Optimizing the operation conditions, like enzyme dose, hydrogen peroxide dose, pH, temperature, addition mode, mixing time and flocculation time, should be studied in order to optimizing the operation cost in the real situation.

The reactions products disposal should be studied in the future studies.

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A. Appendix A

Analytical Methods

A.1 Chemicals

The following is a list of the chemicals used during this research and their molecular weights. The list is provided to facilitate the visualization of the calculations of reagent preparation in subsequent subsections of this Appendix.

Chemical	Formula	Molecular Weight (g/mol)
Certified ACS Sodium Bicarbonate	$NaHCO_3$	84.01
Certified ACS Sodium Phosphate Monobasic	$NaH_2PO_4 - H_2O$	137.99
Certified ACS Dibasic Anhydrous Sodium Phosphate	Na_2HPO_4	141.96
Certified ACS Potassium Ferricyanide	$K_3Fe(CN)_6$	329.26
Certified ACS Hydrogen Peroxide, 30%	H_2O_2	34.01
4-aminoantipyrine (4-AAP)	$C_{11}H_{13}N_3O$	203.25
Certified ACS Solid Phenol	C_6H_5O	94.11
Certified ACS sodium hydroxide	NaOH	40.00
Certified ACS Sulfuric acid	H_2SO_4	98.08
Certified ACS Silver Sulfate	$Ag_2\ SO_4$	311.80
Certified ACS Mercuric Sulfate	$HgSO_4$	99.0
Certified ACS Potassium Dichromate	$K_2Cr_2O_7$	294.19
Certified ACS Potassium Hydrogen Phthalate	$HOCOC_6H_4COOK$	204.23
Glucose/Dextrose	--	--
Yeast	--	--
Peptone	--	--
Malt	--	--

A.2 *Coprinus cinereus* Peroxidase Activity Assay

A colorimetric assay involving the reaction of excess phenol, 4-aminoantipyrine (4-AAP), hydrogen peroxide (H_2O_2), and rate limiting ARP in a monobasic-dibasic sodium phosphate buffer pH 7.4 was used to measure the amount of active enzyme present in a sample. In this assay, the free radicals combine with 4-AAP to form a red quinoneimine dye that absorbs light at a peak wavelength of 510 nm. The color generation during the

first seconds is proportional to the rate of enzymatic H_2O_2 consumption, which in turn is proportional to the concentration of active enzyme in the sample (Wright, 1995).

The assay consists of 10 mM phenol, 2.4 mM AAP, 0.2 mM H_2O_2, in a 0.1 M monobasic-dibasic sodium phosphate (NaPP) buffer pH 7.4, and the enzyme sample, that must not exceed a concentration of 0.05 U mL^{-1} in order to maintain a linear relationship over the 1-minute duration of this assay.

A.2.1 Reagents

a) 0.2 M monobasic sodium phosphate

Dilute 27.6 g of monobasic sodium phosphate with ultra pure water to make 1 L.

b) 0.2 M dibasic sodium phosphate (anhydrous)

Dilute 28.4 g of dibasic sodium phosphate with ultra pure water to make 1 L.

c) 0.1 M NaPP Buffer (pH 7.4)

Add 19 mL of 0.2 M monobasic sodium phosphate to 81 mL of 0.2 M dibasic sodium phosphate and dilute to 200 mL with ultra pure water.

d) 20 mM Phenol

Dissolve 941.1 mg of phenol to 500 mL of 0.1 M NaPP buffer (pH 7.4).

e) 9.6 mM 4-aminoantipyrine (4-AAP)

Dissolve 390 mg of 4-AAP to 200 mL of 0.1 M NaPP buffer (pH 7.4). Store in refrigerator.

f) 2.0 mM H_2O_2

Dilute 113.4 µL of 30% (w/v) hydrogen peroxide to 100 mL with ultra pure water (10 mM). Mix well and withdraw 20 mL and dilute to 100 mL with ultra pure water (2.0 mM). This reagent has to be prepared daily.

A.2.2 Procedure

The volumes of buffer and enzyme sample should be combined to give a total assay volume of 1 mL. Thus, in a semi-micro cuvette place the following reagents:

- 500 µL of 20 mM phenol.
- 250 µL of 9.6 mM 4-AAP.
- 100 µL of 2.0 mM H_2O_2.
- 0 to 100 µL of 0.1 M NaPP (pH 7.4).
- 50 to 150 µL of enzyme sample.

Upon addition of the ARP, the cuvette has to be immediately covered and inverted at least 3 times prior to being placed in the spectrophotometer. The change in absorbance at 510 nm is monitored for at least one minute following reaction initiation.

A.2.3 Calculations

One unit of activity is defined as the number of micromoles of peroxidase converted per minute at 25 °C and pH 7.4. The enzyme activity is obtained from the average slope (absorbance units per minute) within the linear range. Thus, the activity in the cuvette in units of mol min^{-1} mL^{-1} (i.e. U mL^{-1}) is calculated from:

$$Activity\ in\ cuvette\ (U/mL) = \frac{slope(au/\min)}{6280(au \cdot L/mol)} \times \frac{10^6\ \mu mol}{mol} \times \frac{1\,L}{1000\,mL}$$

where *au* represents activity units, and 6280 $au \cdot L/mol$ relates the color development to peroxide consumption.

Furthermore, the sample activity has to be calculated according to:

$$Sample\ activity\ (U/mL) = Activity\ in\ cuvette\ (U/mL) \times \frac{1000\ \mu L}{vol.sample\ \mu L} \times Dilution$$ [1]

A.3 Total Phenol Assay

This assay measures the concentration of phenol in a sample using a colourimetric method. The phenol in the sample reacts with 2.08 mM 4-AAP in the presence of 8.34 mM potassium ferricyanide, which generates a yellowish color. After waiting for 8 to 10 minutes for total color development, the absorbance is measured at 510 nm. The total concentration of phenol in the cuvette is then determined using a calibration curve (as depicted in Figure 3.2). This assay is a modification of the direct photometric method,

[1] Be aware that the sample activity has to be multiplied by the dilution factor (if any) used to maintain an enzyme concentration below or at the 0.05 U/mL required.

5530 D, which is a standard analytical procedure for phenols (APHA, 1995). This modified assay permits measurement of higher phenol concentrations than the standard method, while using smaller sample volumes, by using higher concentrations of 4-AAP and potassium ferricyanide reagents.

A.3.1 Reagents

a) 0.25 M Sodium bicarbonate ($NaHCO_3$)

Dissolve 21.0 g of sodium bicarbonate with ultra pure water to make 1 L.

b) 83.4 mM Potassium ferricyanide

Dissolve 13.73 g of potassium ferricyanide using 0.25 M of $NaHCO_3$ to 500 mL and store in refrigerator.

c) 20.8 mM 4-aminoatipyrine (4-AAP)

Dissolve 2.114 g of AAP using 0.25 M of $NaHCO_3$ to 500 mL Store in refrigerator.

d) 1.0 mM Phenol

Dissolve 47.055 mg of phenol to 500 mL using 0.1 M NaPP buffer (pH 7.4). With this stock solution make standard solutions ranging from 0 to 1 mM.

A.3.2 Calibration Curve Procedure

Mix the following reagents in the order stated:

- 700 µL of 0.25 M sodium bicarbonate.
- 100 µL of standard.
- 100 µL of 20.8 mM AAP solution.
- 100 µL of 83.4 mM potassium ferricyanide.

The assay sample volume must be 1 mL. The phenol concentration in the semi-micro cuvette must not exceed 0.1 mM. Invert several times the cuvette to mix the reagents and place in the spectrophotometer. Record the absorbance at 510 nm after full color is developed (within 8 to 10 minutes). Perform the measurements in triplicates for all standards. Plot the absorbance versus phenol concentration, calculating the slope of the line by linear regression.

A.3.3 Phenol Assay Procedure

In a semi-micro cuvette combine the following reagents to have a final volume of 1 mL:

- 0 to 700 µL of 0.25 M sodium bicarbonate.
- 100 to 800 µL of sample (not exceeding 0.1 mM of Phenol in cuvette)[2]
- 100 µL of 20.8 mM AAP solution.
- 100 µL of 83.4 mM potassium ferricyanide.

[2] Dilute the sample as required if the concentration of phenol will exceed 0.1 mM in the cuvette.

Wait for 8 to 10 minutes for full-color development and read the absorbance at 510 nm.

A.3.4 Calculations

The concentration of phenol in the cuvette is determined from the calibration curve, and the phenol concentration of the sample is obtained from:

$$[Phenol]_{sample} = [Phenol]_{cuvette} \times \frac{1000\ \mu L}{sample\ volume\ (\mu L)} \times Dilution$$

A.4 Hydrogen Peroxide Assay

Hydrogen peroxide concentration is measured using an end-point colourimetric assay that uses 10 mM phenol and 2.4 mM 4-AAP as ARP substrates at pH 7.4. Hydrogen peroxide must be the limiting substrate, thus the maximum concentration in the cuvette should not exceed 50 μM. When the maximum color is developed, the absorbance at 510 nm is converted to the concentration of hydrogen peroxide in the cuvette using a calibration curve.

A.4.1 Preparation of reagents

a) 0.1 M Phosphate buffer (pH 7.4)

Mix 19 mL of 0.2 M monobasic sodium phosphate with 81 mL of 0.2 M dibasic sodium phosphate and dilute to 200 mL with ultra pure water.

b) 20 mM phenol

Dissolve 941.1 mg of solid phenol to 500 mL of phosphate buffer (pH 7.4).

c) 9.6 mM 4-AAP

Dissolve 390 mg to 200 mL of phosphate buffer (pH 7.4). Store in refrigerator.

d) Stock ARP (1 mg/mL)

Dissolve 10 mg of solid ARP to 10 mL using phosphate buffer pH 7.4. Accuracy in the preparation of this stock solution is not required since the only requisite is excess enzyme to reach the endpoint.

e) 10 mM hydrogen peroxide

Dilute 113.4 µL of 30% w/v hydrogen peroxide to 100 mL with ultra pure water. From this 10 mM stock H_2O_2 make standard solutions with concentrations ranging from 0 to 0.5 mM.

A.4.2 Calibration Curve Procedure

In a semi-micro cuvette place the following reagents:

- 500 µL of 20 mM phenol.
- 250 µL of 9.6 mM 4-AAP.
- 100 µL of stock ARP.
- 50 µL of 0.1 M NaPP buffer (pH 7.4).
- 100 µL of standard peroxide solution.

Invert the cuvette several times and place in the spectrophotometer. Wait until the maximum amount of color has developed and record the absorbance at 510 nm. Repeat the same procedure for all peroxide standards prepared, using triplicate measurements.

Note that the assay sample volume must be 1 mL and that the peroxide concentration in the cuvette must not exceed 50 μM. Make the calibration curve by plotting absorbance versus peroxide concentrations in the cuvette.

A.4.3 Assay Procedure

Place the following reagents in a semi-micro cuvette for a final volume of 1 mL:

- 500 μL of 20 mM phenol.
- 250 μL of 9.6 mM 4-AAP.
- 100 μL of stock enzyme.
- 0 to 100 μL of 0.1 NaPP buffer (pH 7.4).
- 50 to 150 μL of sample.

Measure the maximum absorbance at 510 nm. Since the peroxide concentration should not exceed 50 μM, use more NaPP buffer and less sample volume if necessary (or dilute the sample as required) but always maintaining a total volume of 1 mL. The H_2O_2 concentration in the cuvette can be thus obtained from the calibration curve shown in Figure 3.3.

A.4.4 Calculations

The peroxide concentration in the sample is calculated from:

$$[H_2O_2]_{sample} = [H_2O_2]_{cuvette} \times \frac{1000\ \mu L}{Sample\ volume\ (\mu L)}$$

A.5 Glucose Assay

Concentration of glucose in culture media was determined by a colorimetric assay based on the method described in the glucose (GO) assay kit technical bulletin provided by Sigma. Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to forma colored product. Oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the pink color measured at 540nm is proportional to the original glucose concentration.

A.5.1 Reagents

1) Glucose Oxidase/Peroxidase Reagent

Store the unopened kit reagent at 2-8°C. Each capsule contains 500 units of Glucose Oxidase (Aspergillus niger), 100 Purpurogallin units of peroxidase (horseradish) and buffer salts. In an amber bottle, dissolve the contents of the capsule in 39.2ml of deionized water. The solution is stable up to one month at 2-8°C and for at least 6 months frozen at -20°C. discard if turbidity develops.

2) o-Dianisidine Reagent

Store the unopened kit reagent at 2-8°C. Minimize exposure to light. Preweighted vial contains 5mg of o-dianisidine dihydrochloride. Reconstitute the vial of o-dianisidine with 1.0ml of deionized water. Invert the vial several times to dissolve. Avoid exposing the reagent to light. Solution is stable for 3 months at 2-8°C.

3) Assay Reagent

Add 0.8ml of the o-Dianisidine Reagent to the amber bottle containing the 39.2ml of Glucose Oxidase/Peroxidase Reagent. Invert bottle several times to mix. Minimize exposure to light. Solution is stable up to 1 month at 2-8°C. discard if turbidity develops or color forms

4) Glucose Standard Solution

D-Glucose, 1.0mg/ml in 0.1% benzoic acid. Store reagent at 2-8°C. Supplied ready to use. Solution is stable at 2-8°C for at least six months. Discard if turbidity develops.

5) Sulfuric Acid (12N)

Diluted 82.85 mL 95.0 – 98.0 % concentrated sulphuric acid to 250 ml using pure water.

A.5.2 Calibration Curve Procedure

1) Pipette the following solutions into the approximately marked test tubes:

Tube	Water (mL)	Sample (mL)	Glucose Standard (mL)
Reagent Blank	1.00	--	--
Standard # 1	0.98	--	0.02
Standard # 2	0.96	--	0.04
Standard # 3	0.94	--	0.06
Standard # 4	0.92	--	0.08
Test	--	1.00	--

2) At zero time, start the reaction by adding 2.0ml of Assay Reagent to the first tube and mixing. Allow a 30 to 60 second interval between additions of Assay Ragent to each subsequent tube.

3) Let each tube react exactly 30 minutes at 37°C in the water bath. Stop the reaction at 30-60 second intervals by adding 2.0ml of 12 N H2SO4 into each tube.

4) Carefully mix each tube thoroughly, measure the absorbance of each tube against the reagent blank at 540nm.

A.5.3 Glucose Assay

1. Pipette the following solutions into the approximately marked test tubes:

Tube	Water (mL)	Sample (mL)	Glucose Standard (mL)
Regent Blank	1.00	--	--
Standard	0.95	--	0.05
Test	--	1.00	--

2. At zero time, start the reaction by adding 2.0ml of Assay Reagent to the first tube and mixing. Allow a 30 to 60 second interval between additions of Assay Reagent to each subsequent tube.
3. Let each tube react exactly 30 minutes at 37°C. Stop reaction 30-60 second intervals by adding 2.0ml of 12 N H2SO4 into each tube. Carefully mix each tube thoroughly.
4. Measure the absorbance of each tube against the reagent blank at 540nm.

A.5.4 Calculations

$$[Glucose]_{sample} = [Glucose]_{cuvette} \times \frac{1000\ \mu L}{Sample\ volume\ (\mu L)} \times Dilution$$

APPENDIX B

B.1 *Coprinus cinereus* peroxidase production (CIP) data

Table B-1: Glucose Standard Curve data

Glucose Conc. mg/L	Abs
0	0.003
60	0.874
80	1.223
100	1.5

Table B-2 Batch CIP production data

Time, Day	Enzyme Activity U/mL	Glucose g/L	pH
0	0	10	5. 99
1	0. 123	9. 974	6. 16
2	0. 1975	9. 073	5. 9
3	0. 3634	8. 446	5. 63
4	0. 5172	5. 841	5. 94
5	1. 4814	3. 748	5. 59
6	8. 8851	2. 517	5. 02
7	19. 19	0. 034	6. 21
8	18. 931	0. 02	7. 38
9	15. 954	0. 004	7. 63
10	11. 184	0. 004	7. 98

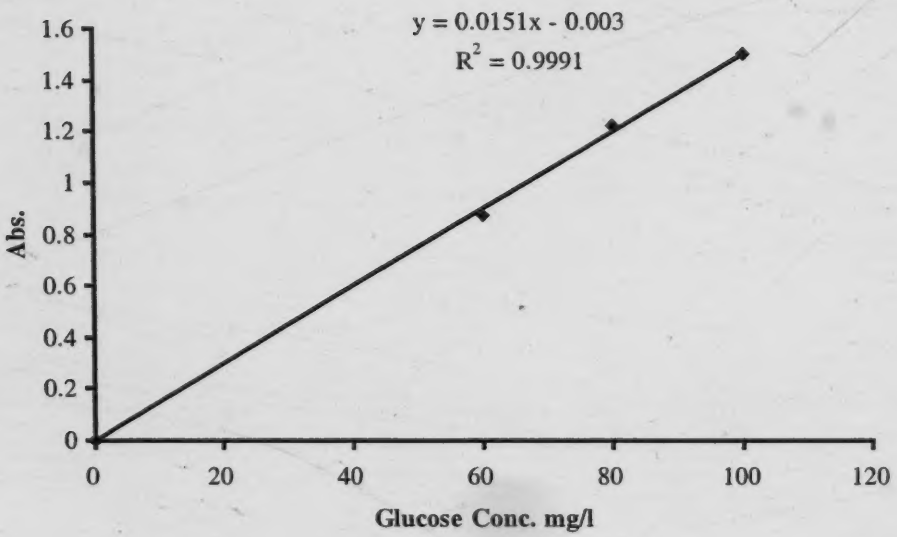

Figure B-1: Glucose standard Curve

Table B-3: Semi-batch CIP production data

Time, day	Enzyme Activity U/mL	Glucose g/L	pH
0	0	10	5.94
1	0.198	9.695	5.97
2	0.268	9.338	5.5
3	0.683	8.464	5.06
4	1.686	6.755	5.71
5	4.898	4.517	5.99
6	8.954	1.554	6.02
7	12.394	0.141	6.3
8	13.663	0.0933	7.2
9	12.923	0.015	7.84
10	10.827	0.004	8.11
11	--	--	--
12	2.966	6.53	5.72
13	16.785	2.159	6.49
14	24.269	0.311	6.95
15	31.282	0.008	7.36
16	20.887	0.005	7.84
17	--	--	--
18	6.2138	5.934	5.85
19	11.201	2.278	6.38
20	10.379	0.284	6.49
Stopped due to agitation problem			

Table B-4: Enzyme production data in shaker flasks in a media with or without malt

	Glucose conc, g/L		Enzyme activity, U/mL	
Time, day	With Malt	Without Malt	With Malt	Without Malt
6	7.013	7.06	1.00	1.00
8	1.94	2.457	12.48	11.00
10	0.00543	0.00503	18.15	17.72
11	0.00457	0.00443	22.20	20.21

Table B-5: Enzyme production data in a batch fermentor

Time, day	Enzyme Activity U/mL	Glucose g/L
0	0	20
6	18.7828	17.351
7	26.363	13.43
8	41.846	3.377
9	58.99	0.16
10	56.71	0.057

B.2. *Coprinus cinereus* peroxidase purification data

Table B-6: Enzyme activity after vacuum filtration through different membranes

Filter Type	Enzyme activity, U/mL
Q8	13.179
0.45 um	13.02
0.22um	12.856

Table B-7: Enzyme activity in Permeate and retentate of various membranes

Filter Type	Enzyme, U/mL
0.22um	5.093
0.22um	5.076
100k P	4.85
100k P	4.87
100k R	4.99
100k R	4.943
10k P	0.276
10k P	0.269
10k R	5.161
10k R	5.17

Table B-8: COD value in permeate and retentate of various membranes

Filter Type	COD mg/L
0.22um	4500
0.22um	4598.4
100k P	4336
100k P	4286.8
100k R	4204.8
100k R	4090
10k P	3680.4
10k P	3713.2
10k R	4614.8
10k R	4500

Table B-9: COD standard Curve

COD, mg/L	Abs
0	0
200	0.049
200	0.049
200	0.049
400	0.098
400	0.098
400	0.098
600	0.147
600	0.146
600	0.144
800	0.196
800	0.197
800	0.195
1000	0.249
1000	0.246
1000	0.245
1200	0.287
1200	0.292
1200	0.292

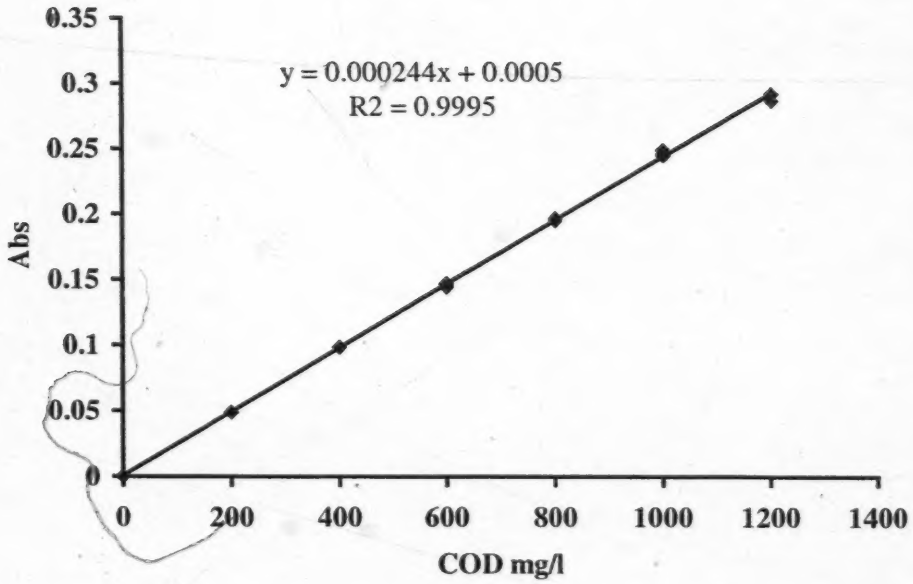

Figure B-2: COD Standard Curve

B.3. Batch reactor data

Table B-10: Phenol removal efficiency at different filtrate and retentate

Filter Type	Phenol Conc. mg/L	Removal efficiency, %
Orignal	0.115	0.885
Q8	0.131	0.869
0.45 um	0.137	0.863
0.22 um	0.139	0.861
100k P	0.145	0.855
100k R	0.150	0.850
10k R	0.148	0.852

Table B-11: Phenol standard curve data

Phenol concentration mM	Absorbence
0	0.003
0	0.003
0	0.003
0.02	0.232
0.02	0.233
0.02	0.232
0.04	0.466
0.04	0.465
0.04	0.466
0.06	0.693
0.06	0.695
0.06	0.693
0.08	0.905
0.08	0.934
0.08	0.934
0.1	1.148
0.1	1.158
0.1	1.148

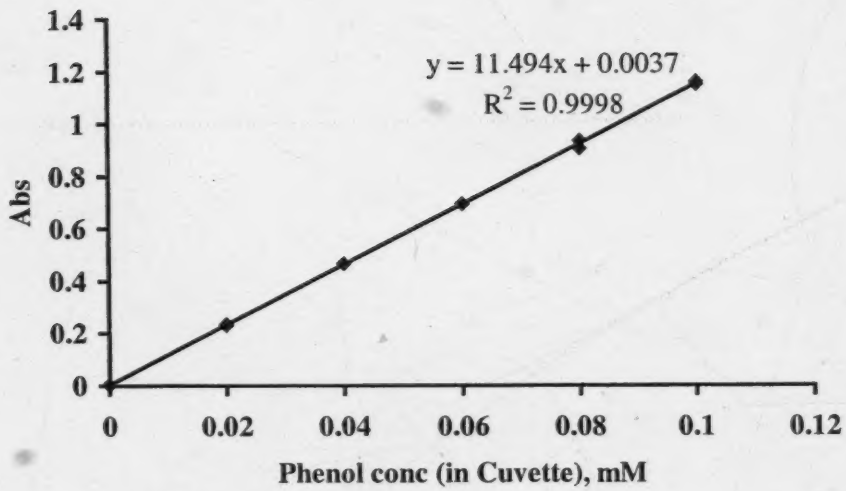

Figure B-3: Phenol standard curve

Table B-12: Comparison of the phenol removal by ultrafiltrated and purified enzyme

Removal, %	Crude Enzyme	Pure enzyme
N	86.7	92.4
P	90.2	95.5
B	50.0	17.9
B+P	95.2	97.9

Table B-13: Hydrogen peroxide standard curve data

H_2O_2 Conc. mM	Abs
0	0.05
0	0.049
0	0.049
0.01	0.11
0.01	0.11
0.01	0.111
0.02	0.164
0.02	0.166
0.02	0.165
0.04	0.268
0.04	0.269
0.04	0.269
0.06	0.379
0.06	0.381
0.06	0.381
0.08	0.491
0.08	0.492
0.08	0.491
0.1	0.592
0.1	0.592
0.1	0.593

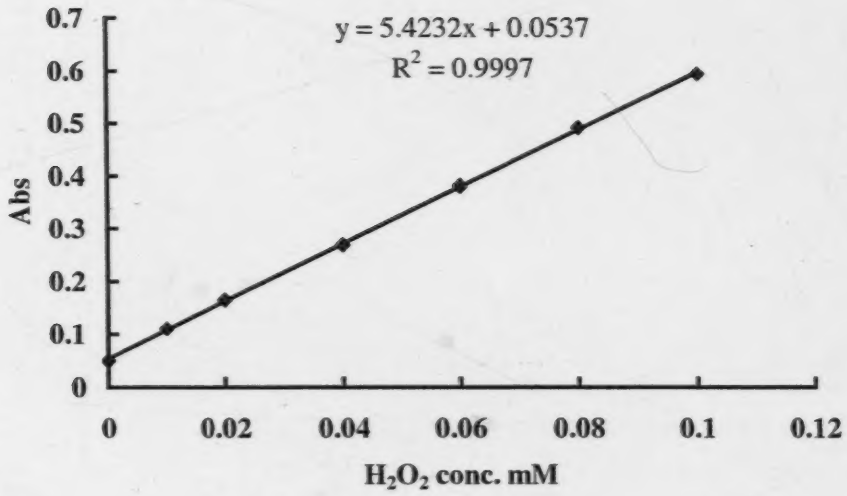

Figure B-4: Hydrogen Peroxide standard curve

B.4. Plug flow reactor data

Table B-14: Time courses of phenol removal at pH7.0, $[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, $[CIP]_0$ = 0.65 U mL^{-1}, $[PEG]_0$ = 200 mg L^{-1}

	Phenol Removal, %	
Rt,min	0.2g/L PEG	No PEG
0.00	0	0.0
0.00	0	0.0
7.89	25.2	40.5
7.89	26.2	40.6
15.79	42.4	52.2
15.79	41.3	52.1
31.58	49.4	60.0
31.58	49.6	59.9
60.00	56.2	65.7
60.00	56.5	65.8

Table B-15: Time courses of COD Removal at pH7.0, $[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, $[CIP]_0$ = 0.65 U mL^{-1}, $[PEG]_0$ = 200 mg L^{-1}

	COD remaining, mg/L	
Rt, min	0.2g/L PEG	No PEG
0.00	600.4	248.0
0.00	592.2	243.9
7.89	567.6	166.0
7.89	563.5	161.9
15.79	506.1	137.3
15.79	485.7	149.6
31.58	440.6	120.9
31.58	440.6	108.6
60.00	448.8	88.1
60.00	461.1	84.0

Table B-16: Phenol removal at pH7.0, $[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, [total CIP] = 0.65 U mL^{-1}, 0m 0.45 U mL^{-1} &4m 0.2 U mL^{-1} enzyme

	Phenol removal, %	
Rt, min	0.2 g/L PEG	No PEG
0.00	0	0
0.00	0	0
7.89	51.7	54.2
7.89	51.7	54.2
15.79	61.4	63.5
15.79	60.9	63.1
60.00	72.3	80.3
60.00	73.0	80.3

Table B-17: Phenol removal at pH7.0, $[Phenol]_0$ = 1 mM, $[CIP]_0$ = 0.65 U mL^{-1}, [total H_2O_2] = 1.1 mM, 0 & 4m 0.8 & 0.3mM H_2O_2

	Phenol removal, %	
Rt, min	0.2g/L PEG	No PEG
0.00	0	0
0.00	0	0
7.89	44.6	59.7
7.89	44.9	59.2
15.79	51.1	66.0
15.79	51.5	65.7
60.00	68.3	72.1
60.00	68.4	72.2

Table B-18: Phenol removal with different CIP concentration at pH7.0,

$[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM

	Phenol Removal %	
Rt, min	**0.65U mL^{-1}**	**1.5U mL^{-1}**
0.00	0	0.0
0.00	0	0.0
7.89	41.5	85.7
7.89	42.1	85.2
15.79	67.9	89.7
15.79	68.4	89.7
31.58	69.8	89.6
31.58	69.8	89.6
60.00	64.8	90.0
60.00	65.2	89.7

Table B-19: Phenol removal with step addition of CIP at pH7.0,

$[H_2O_2]_0$ = 1.1 mM, $[Phenol]_0$ = 1 mM, [total CIP] = 0.65 U mL^{-1}

	Phenol removal, %		
Rt, min	**0m 0.65U/mL**	**0m 0.33U/mL 4m 0.33U/ mL**	**0m 0.45U/ mL 4m 0.2U/ mL**
0.00	0.0	0.0	0.0
0.00	0.0	0.0	0.0
7.89	40.5	33.7	54.2
7.89	40.6	33.1	54.2
15.79	52.2	47.8	63.5
15.79	52.1	47.8	63.1
60.00	65.7	70.4	80.3
60.00	65.8	70.4	80.3

Table B-20: Phenol removal with step addition of H_2O_2 at pH7.0,

$[Phenol]_0 = 1$ mM, $[CIP]_0 = 0.65$ U mL^{-1}, [total H_2O_2] = 1.1 mM.

	Phenol removal, %	
Rt, min	0m 0.8mM 4m 0.3 mM H_2O_2	0m 1.1mM H_2O_2
0.00	0	0
0.00	0	0
7.89	59.7	40.5
7.89	59.2	40.6
15.79	66.0	52.2
15.79	65.7	52.1
60.00	72.1	65.7
60.00	72.2	65.8